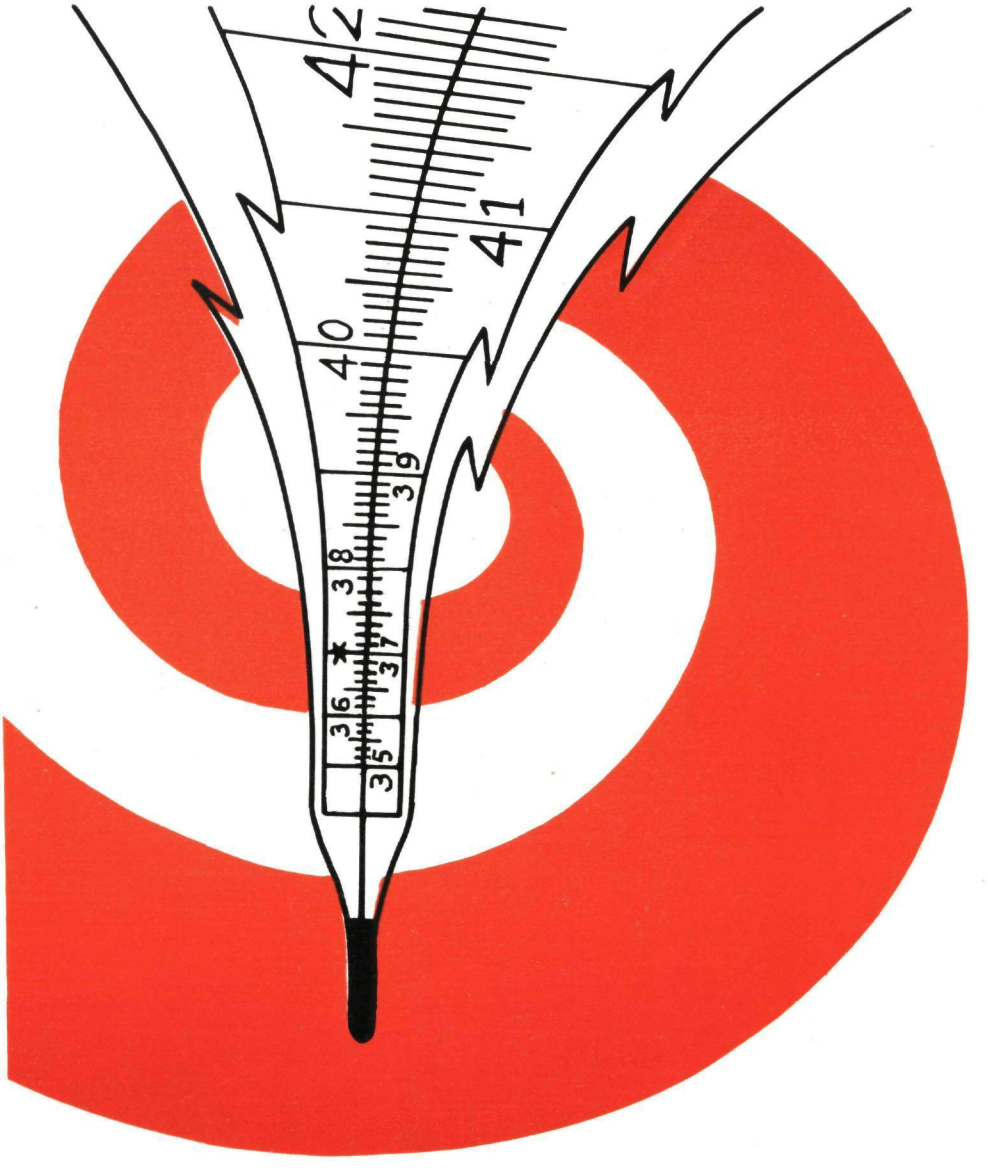
M.P. VERBURG

MALIGNANT HYPERTHERMIA

THE ANAESTHESIA INDUCED DISEASE



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MALIGNANT HYPERTHERMIA

THE ANAESTESIA INDUCED DISEASE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS, PROF DR J H G I GIESBERS VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG 24 MEI 1984 DES NAMIDDAGS TE 2 00 UUR PRECIES

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MARINUS PIETER VERBURG

GEBOREN TE GEERSDIJK

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CONTENTS :

ABBREVIATIONS

AMP, ADP, ATP: Adenosine 5´-mono-, di-, and triphosphate.

CK: Creatine kinase.

Cr: Creatine.

Hyx: Hypoxantine.

IMP: Inosine monophosphate.

LDH: Lactate dehydrogenase.

MH: Malignant hyperthermia.

NAD^+, NADH: nicotinamide adenine dinucleotide oxydized, - reduced.

PCr: Phosphocreatine.

Pi: Inorganic phosphate.

CHAPTER 1 : INTRODUCTION

Malignant hyperthermia (MH) is a pharmacogenetic disorder, characterized by an induced hypermetabolism accompanied with a high mortality[1,2]. An MH crisis presents clinically by a rapid increase of body temperature, skeletal muscle rigidity and cardiovascular symptoms like tachycardia and instable bloodpressure. The final symptoms are coma and cardiovascular collaps. The appearance of these symptoms may develop fulminantly (increase of body temperature by about 1 °C every 5 min), or slowly, during the surgical procedure or later on in the recovery room [1,2].

An MH crisis is induced in the genetically predisposed subjects by depolarizing skeletal muscle relaxants (succinylcholine) or volatile anaesthetics (ether, ethrane, halothane, forane, isoflurane)[1,2]. MH is considered to be an autosomal-dominantly inherited disorder, with variable expression and reduced penetrance[1,2]. Due to this heridity and the high mortality, a family screening is indicated in those families in which an MH crises has occurred. The overall incidence of MH is thought to range from 1/50,000 to 1/100,000 anaesthesias administered to adults, and about 1/15,000 in children[2]. This means, that every anaesthetist may meet only 1 to 2 MH crises during his or her professional life. This rarety contributes to the high mortality. Furthermore, some people develop an MH crisis although they have had several uneventful

anaesthesias before, even with MH triggering agents[1,2]. This contributes to the confusion regarding the understanding of the syndrome.

As the triggering agents are commonly used drugs in anaesthetic practice, it is of importance for the anaesthetist to be informed about the clinical and pathophysiological effects of these drugs in MH susceptible subjects. Since the clinical and pathophysiological features of MH are manifestations of the induced biochemical changes, it is of importance to understand the basical biochemical processes regarding the energy production and consumption, as well as external factors influencing these processes. Especially the role of calcium is important, as it has been hypothesized that an increased concentration of calcium in the myoplasma accounts for the skeletal muscle rigidity and the disturbance of energy production and energy consumption[1,2]. These aspects are extensively reviewed in chapter 2 and 3 of this thesis.

Furthermore, this literature review was felt necessary, as considerable conflicting data have reported regarding the aetiology of MH, especially the role of the subcellular components of skeletal muscle. The mitochondria are the sites of aerobic energy production and calcium has a detrimental effect on the energy producing processes[3]. Another important sub-cellular component in skeletal muscle is the sarcoplasmic reticulum (SR), regulating the physio-

logical concentrations of intra-cellular calcium ion concentrations. The cellular membrane of skeletal muscle, sarcolemma, acts as a barrier between the high extra-cellular calcium ion concentration and the low intra-cellular one. As a disturbance in the functioning of these organelles has serious repercussions on the whole cell, these aspects are discussed in chapter 3.

A review of the present and previous screening methods is given in chapter 4. This is done because a wide variety exists in screening methods and the current method is used in the experimental part of the thesis. Wereas the first part of the thesis, chapter 2, 3 and 4, is a review of the existent literature, it also forms the background for our experimental work, which is described in the second part of this thesis, chapter 5-10.

This experimental part can be divided in two sections: the first one concerning experiments on MH susceptible pigs and control pigs (chapter 5, 6 and 7). These experiments were performed, based on the following questions:
I: What is the speed of pathophysiological changes observed during an MH crisis, and can a useful specific parameter be found as an early indicator of MH?
II: What happens in energy metabolism during an MH crisis, especially in skeletal muscle metabolism and mitochondrial functioning ?
III: Can an indication be found for the role of calcium

in the aetiology of MH, especially an increased concentration of calcium in the myo-plasma or its organelles? IV: Is dantrolene sodium effective as a curative drug, and what is its effect on energy metabolism and calcium flux?

The second section of our experimental work is a condensation of proposed and existing screening methods of MH susceptibility performed on humans. Chapter 8 is an evaluation of an advocated "non-invasive" screening technique, using platelets [4]. The aim of our study was to verify this test as a possible screening method. Chapter 9 is an evaluation of skeletal muscle metabolites in humans susceptible to MH and controls. This study was performed on the basis of the results of our experiments on pigs. The question to be answered was: can skeletal muscle metabolite analysis be used as a screening method to detect MH susceptibility? Chapter 10 gives a summary of the results in our hands of the present MH screening test: the socalled in-vitro caffeine contracture test (CCT). As our laboratory is the first one to perform this test in the Netherlands, we thought it indicated to describe the methods used in full. The test procedure used, is in accordance with the concept of a uniform test procedure in Europe, as described in the appendix of this thesis.

The final chapter 11, reconciles the theoretical considerations of part one with the experiments work of

part two of this thesis. Severa recommendations are made concerning testing for MH susce tibility in anaesthesiological practice and early reco nition of an MH crisis.

Literature :

1. Gronert G A. Malignant hyper hermia. Anesthesiology 1980: 53, 395-423.
2. Nelson T E, Flewellen F H. C rrent concepts: malignant hyperthermia. New Engl J Med 1983: 309, 416-418.
3. Lehninger A L. Biochemistry 2nd ed, Worth 1977: 531-533.
4. Solomons C A, McDermott N, Mahowald M. Screening for malignant hyperthermia with a platelet bio-assay. New Engl J Med 1980: 303, 642.

INDEX CHAPTER 2 :

Malignant hyperthermia as a clinical syndrome

2.1 CLINICAL SYMPTOMS

The first recognition of MH as a clinical syndrome dates back to 1960, when Denborough and Lovell described MH as a clinical entity and its heriditary aspect[1]. From that time on, MH has been considered as a pharmacogenetic disease, i.e. exposure to certain drugs will initiate MH in an otherwise healthy subject. The most marked symptoms of MH are skeletal muscular rigidity and a rapid increase of body temperature[2].

2.1.1. Muscle rigidity

Skeletal muscle rigidity is often noted immediately after administration of a depolarizing muscle relaxant like succinylcholine. This rigidity is sometimes preceded by fasciculations, stronger than usually observed.
The rigidity is most markedly present in the jaw[3].
This may prevent tracheal intubation, or will make it extremely difficult. In case of emergency surgery or in a patient with a full stomach, this may lead to aspiration of gastric contents: a catastrophic situation for the patient. On the other hand, muscular rigidity may develop during the surgical procedure, even when succinylcholine was used without problems at the beginning of the procedure[4].

Furthermore, muscular rigidity after administration of succinylcholine may also occur if the patient is suffering

from some other muscular disease[5].

In cases of muscular dystrophy or myotonia congenita, generalized muscular rigidity may occur after administration of succinylcholine, but no hyperthermic response will ensue, and the muscular rigidity will fade after metabolism of succinylcholine, i.e. after about 4-5 minutes[5]. Other myopathies may also produce muscular rigidity: polymyositis, mitochondrial myopathies, demyelinating disorders and chronic or partial denervation of skeletal muscle[5].

Muscular rigidity may also develop in MH after using non-depolarizing muscle relaxants from the very beginning. During the surgical procedure this may become apparent, and the request by the surgeon for increased muscle relaxation should only be honoured if the possibility of MH has been excluded. This rigidity mostly occurs in muscle of the limbs, making joint movements practically impossible. Ultimately this rigidity may hamper also the ventilation of the patient. The rigidity in the final stage of MH is quite similar to rigor mortis.

2.1.2. Temperature elevation

It has been claimed that continuous temperature monitoring in anaesthesia is essential to recognise an MH crisis in a very early phase[4]. This statement should be viewed with scepticism. In a study of over 2,400 patients receiving general anaesthesia, it was noted that 10-20% of the patients developed a temperature increase during short surgical

procedures[6]. This happened in patients, arriving in the operation theatre with an already decreased core temperature. Also the use of depolarizing muscle relaxants and halothane produced significantly ($p < 0.05$) higher temperatures than patients receiving non-depolarizing muscle relaxants or no halothane at all. All the patients studied had no family history of MH, nor did they develop any signs of MH during the anaesthetic procedure or in the recovery room. The conclusion from this study is that temperature elevation during general anaesthesia may be a physiological response in patients, arriving for operation with a lowered body temperature[6]. Generally, body temperature will decrease during an operation.

An increase of body temperature depends on the amount of energy produced, the caloric properties of the mass to be warmed, and the loss of heat to the environment. The increase of mass temperature is a time dependent process. The specific heat of human tissue is about 1 Kcal/kg body weight. So, once the body temperature has increased 2 or 3 degrees centigrade, the metabolic processes have already produced in the "standard human being", weighing 70 kg, at least 140-210 Kcal of heat. This excessive heat production can only originate from a strongly increased metabolism, which is evident by the increased oxygen consumption, the oxygen desaturation and increased carbon dioxide pressure of the central venous blood.

Furthermore, anaerobic metabolism will contribute to the

energy production, evident from the increased lactate production during an MH crisis. An increase of body temperature, fast enough to be noted quickly, still needs 15-30 minutes before one can conclude the presence of an MH crisis. In the meantime, tissue damage will occur, due to the severe acidosis and hypoxia.
Consequently, the measurement of body temperature is rather useless as an early warning signal due to the relatively long time period needed to increase body temperature to pathological levels.

2.1.3. Cardio-vascular symptoms

Another symptom of MH is the unstable cardio-vascular system during the crisis. Periods of hypertension and hypotension may be present, both with the occurrence of cardiac arrythmias, mostly ventricular premature beats.
These signs are, however, not specific for MH and may be present in the case of inadequate analgesia, traumatic surgery causing increased sympathetic reactions, or exaggerated bloodloss. The occurrence of tachycardia, frequently observed in MH, is considered by some authors as a consistent early sign of MH[7]. However, this is a rather non-specific sign and very frequently observed at the start of an operation using general anaesthesia, most often caused by increased sympathetic stimulation or a low filling grade of the vascular system. Also a drop in bloodpressure after induction of anaesthesia, will evoke compensatory tachycardia. The observation

of tachycardia during a surgical procedure, may be suggestive of the development of MH, provided that no other causes are present such as excessive surgical stimulation or cardiovascular instability. The occurrence of cardiac arrythmias in the course of a surgical procedure is very unlikely to be due to "light" anaesthesia, unless the vaporizer has run dry if an inhalation anaesthetic is used. In the full blown MH crisis, besides tachycardia, also ventricular arrythmias will occur. The ECG complex may change in the course of of the MH attack due to hyperkalaemia, producing tall peaked T waves [7].

2.1.4. Respiratory symptoms

Tachypoea during surgery when the patient is breathing spontaneously or, spontaneous breathing efforts while the patient is ventilated, is a sign to be taken seriously. Besides the possibility of MH it may be caused by a low F_iO_2 or decreased alveolar ventilation, e.g. defaulted ventilator or disconnection of the tubings.
This is a non-specific phenomenon of MH and only a symptom of inadequate oxygenation of the tissues.

2.1.5. Miscellaneous symptoms

The occurrence of left ventricular failure and pulmonary oedema are signs of MH in its late phase. When these signs appear, mortality will be very high: 70-100% [3].
In this phase of MH the therapeutic administration of dantrolene will usually be in vain. Due to the circulatory

failure, there will be a poor tissue perfusion and a marked periferal vasoconstriction caused by the increased catecholamine levels.
At this high temperature protein changes will occur, leading to the activation of the coagulation cascade, causing intravascular coagulation.

Furthermore, renal complications will occur due to rhabdomyolysis[4]. Liberated myoglobine from damaged skeletal muscle will form precipitations in the renal tubules, leading to a further deterioration of renal function or shut down.

Neurological complications may be quite severe, caused by tissue hypoxia, acidosis and the extremely high temperature[7]. This is presented in the fullblown MH case as coma, leading to cerebral death. In patients recovering from MH, major or minor brain damage will ensue. In these cases, considerable social and psychological damage to the patient may lead to medico-legal suits.

2.2 BIOCHEMICAL CHANGES

2.2.1. Bloodgas changes

The most marked changes include acidosis, hypercarbia and oxygen desaturation of the arterial blood as well as the central venous blood[8,9,10]. The acidosis is both of metabolic and respiratory origin. The major organ producing these changes is skeletal muscle. As skeletal muscle contributes to about 40% of the bodyweight, it will be clear that a disorder in metabolism of skeletal muscle will induce considerable physiological disturbances. The speed of these metabolic changes after initiation of the MH crisis is frightening. Based on the observations of the porcine MH model, pH may drop within five minutes from the resting level of 7.4 to 6.8 or below[8,9]. Furthermore, carbon dioxide production increases several times, as well as oxygen consumption. These changes are best reflected on gas analysis of the central venous blood[9,10]. However, in minor surgical procedures, no indwelling central venous line is used, so this information is not available.

Unexplained acidosis with increased carbon dioxide pressure and decreased oxygen pressure, shown by arterial gas analysis, are diagnostic signs of MH[7,8,9,10].

2.2.2. Metabolic changes

Metabolic changes in MH have been studied extensively in the pig model[9,10,11]. The most remarkable

change is the rapid decline of pH value in skeletal muscle of MH susceptible pigs during the MH crisis, or immediatly after death[4]. In fact, the rapid decline of pH and the associated development of pale soft excudative pork (PSE) have stimulated the veterinarians to study this syndrome extensively. PSE-pork has an excessive loss of water after slaughter making this meat unsuitable for human consumption. The rapid decline in pH is an early change and can be detected in the venous blood draining the hindlimb or in the central venous blood[4,8]. The pH value is also significantly decreased in the arterial blood[8,10,11].

Increased concentrations of lactate have been measured in skeletal muscle during the MH crisis, and also in the venous blood draining skeletal tissue masses[8,9,10,11]. Lactate concentration is also elevated in the arterial blood and analysis of the lactate content of the arterial blood will confirm the MH crisis[8,9]. However, the determination of lactate is a time consuming laboratory technique, and in case of emergency not as useful as the arterial bloodgas analysis. Lactate in skeletal muscle originates from glycogen breakdown. This has been shown in the pig model[11,12]. In muscle of MH susceptible humans and pigs, pre-existently increased levels of glucose-6-phosphate (G-6-P) have been detected[13,14]. This may point to an increased glycolytic activity or to the existence of a futile cycle. Such a futile cycle has been proposed

as a factor in the aetiology of MH. This means that the final result will be the hydrolysis of ATP to ADP and Pi. The reaction mechanism is as follows :

glucose + ATP $\longrightarrow$ G-6-P + ADP

G-6-P + H_2O $\longrightarrow$ glucose + Pi + heat.

However, no firm evidence has emerged in favour of the existence of this futile cycle in the course of further studies on MH in pigs and humans[12].

The process of futile cycling is anaerobic. During MH, however, oxygen consumption increases several times, as compared to the control state[8,9]. This makes a major role of futile cycling of G-6-P unlikely.

2.2.3. Oxygen consumption

Various investigations have been performed on the role and extent of oxygen consumption during MH in pigs. After induction of MH with either halothane alone or in combination with succinylcholine, a significant drop in arterial P_{O_2} was observed, indicating increased oxygen consumption[8,9,10]. Starting in the prechallenge period with a basal total body oxygen consumption of 3-4 ml O_2 STPD/min/kg BW, oxygen consumption increased during the challenge to about 20 ml O_2 STPD/min/kig BW[15]. Studies on isolated perfused hindlimbs showed a basal oxygen consumption of about 2 ml O_2 STPD/min/kg muscle mass, increasing to more than 20 ml O_2/min/kg muscle mass during the MH crisis [16]. Based on the observed oxygen consumption of skeletal muscle mass, one can calculate the contribution

of oxydative metabolism to the increase of body temperature. Because 1 liter of oxygen at STPD produces 5.05 kCal at oxidation of glucose, and the specific heat of the body is about 0.85 kCal/kg/^{o}C, one can calculate the temperature rise .

$$\text{temperature increase/kg muscle/min} = \frac{O_2 \text{ consumption STPD} \times 5.05}{0.85}$$

$$\text{temperature increase/kg muscle/min} = \frac{0.02 \times 5.05}{0.85}$$

$$\text{temperature increase/kg muscle/min} = 0.11\ ^{o}\text{C}.$$

A temperature increase of 1 ^{o}C/5 min in skeletal muscle can be ascribed for about 50% to oxydative metabolism. Anaerobic metabolism will contribute additional energy, needed to increase skeletal muscle temperature with 1 ^{o}C/5min/kg muscle.

2.2.4. Carbon dioxide production

During the MH crisis an increased production of CO_2 occurs, simultaneously with the increased oxygen consumption. The respiratory quotient (R.Q.) during the MH crisis ranges from 1.1 - 1.4[8,9,10,11] . Carbon dioxide concentration is most markedly increased in the venous blood draining muscular masses, like the hind limbs[9]. The increased CO_2 pressure is reflected in the central venous blood. Both in pigs[8,9,10,11,16] and in humans[18] an increased CO_2 pressure in the arterial blood has been observed during the MH crisis. Therefore, arterial bloodgas analysis is a valuable procedure to confirm the diagnosis of MH.

However, it is still a time consuming procedure, taking at least 5-15 minutes to obtain the results after taking an arterial blood sample. During the MH crisis this means loss of valuable time before starting treatment.

2.2.5. Serological changes

2.2.5.1. Serum electrolyte changes

The most marked electrolyte change is the increased K^+ concentration in the venous and arterial blood during an MH crisis in humans as well as in pigs. Still a debate exists about the origin of the K^+ ions. Some authors having studied MH in pigs state that skeletal muscle is the main origin of the increased K^+ concentration[9,11]. Others claim that the liver is the main site of production of the increased K^+ ions[19].

Other electrolyte changes like increased serum Ca^{2+} and Mg^{2+} ion levels, are relatively late events during an MH crisis, and are only significantly increased in the terminal phase of the MH crisis[4] in pigs as well as in humans.

2.2.5.2. Serum metabolite changes

During the MH crisis an immediate rise of the glucose concentration can be observed. The increased glucose production originates from skeletal muscle[20]. It was stated that the liver is the main organ in producing glucose[19], but on additional experiments, contradictory

results have been presented, excluding the role of the liver in producing glucose[4]. Increased efflux of lactate, pyruvate and alanine has been observed from skeletal muscle during an MH crisis in pigs [20].

2.2.5.3. Serum enzyme changes

Other major changes in serological parameters - in humans and pigs - are the increased concentrations of the enzymes creatine phosphokinase (CK) and lactate dehydrogenase (LDH)[4]. These enzymes are liberated from skeletal muscle during the MH crisis[4]. CK concentrations may be elevated already pre-existently in MH susceptible humans and pigs (see also chapter 3). However, marked increase during the MH crisis is caused by skeletal muscle damage. This muscular damage leads also to the liberation of myoglobine. Increased serum concentration of myoglobine may produce renal shutdown, especially when the patient has suffered from an episode of severe hypotension.

2.2.6. Skeletal muscle metabolites

During the MH crisis a significant decrease occurs in the concentration of PCr and ATP in skeletal muscle in pigs [12,14]. This is probably due to the increased energy demand and a decreased production capacity for these compounds. Considerable research has been carried out on the pre-existent concentrations of these compounds, the changes during the MH crisis as well as the influence of anaesthetics. Most of these results have been obtained in pig studies. The importance

of studying the effects of anaesthetics on muscle metabolism is based on the observation that the administration of barbiturates[22,23], neuroleptics[23,24] or non depolarising skeletal muscle relaxants delay the onset of an MH crisis in pigs exposed to MH triggering agents.

Studying skeletal muscle metabolism in animals, one has to take into account the red and white fibre compositon of the muscle. The distribution of these two fibre types is different in various types of skeletal muscle. This in contrast to human skeletal muscle, showing an average 50/50 distributionin limb muscle. The red fibre types are predominantly oxydative in their metabolism, the white one's glycolytic (i.e. anaerobic).
No significant differences in PCr content have been measured between white- and red fibre types, in the same breed of pigs[21,22]. However, PCr concentrations were all significantly lower in MH susceptible pigs compared to control pigs[22]. (see table I).
All these samples have been taken during pentobarbital anaesthesia[22].

Table I :
Mean (± SEM) concentrations of intramuscular phosphocreatine (PCr) and adenosine triphosphate (ATP) in m.semitendinosus of control and malignant hyperthermia (MH) susceptible pigs. Values in umoles/g wet weight, mean ± SEM.

	Control pigs (n=5)		MH susceptible pigs (n=8)	
	red fibre	white fibre	red fibre	white fibre
PCr	28 ± 3	33 ± 4	14 ± 3	18 ± 3
ATP	9 ± 1.5	8.6 ± 1	6.6 ± 1	7.2 ± 1

From: Ahern C P et al. J Comp Path 1980: 90, 177-186.

On continuation of this experiment, halothane was administered, and muscle samples were taken from the red and white fibre portion of the m.semitendinosus in MH susceptible pigs and controls [22]. In MH susceptible pigs a sharp drop was observed in PCr content in both the red and white fibre type muscle. Just prior to death, PCr concentration had dropped to about 5 umoles/g wet weight. In the control pigs, an insignificant drop was measured in PCr content in both fibre types[22]. On exposure to halothane, significantly lower ($p < 0.01$) PCr concentrations were observed in skeletal muscle compared to biopsies obtained during pentobarbital anaesthesia on the same pig[14]. This was observed both in control and MH susceptible pigs, but the decrease in PCr concentration was significantly greater ($p < 0.01$) in MH susceptible pigs than in control pigs[14].

Apparently, pentobarbital has a saving effect on energy rich compounds in skeletal muscle and delays the depletion of energy rich compounds during an MH crisis[22]. Additional studies on skeletal muscle metabolism in pigs have shown a decrease of glycogen concentration during the MH crisis [12,21]. Furthermore, increased concentrations of glycolytic intermediates were measured as well as an increased concentration of glucose in skeletal muscle during the MH crisis[12]. Pyruvate concentration remained unchanged during the MH crisis[12].

2.3 VARIABILITY IN TRIGGERING

A puzzling aspect of MH in humans is the variability of the triggering of an MH crisis by the known MH inducing agents. It is well known that humans, developing an MH crisis may have had several uneventful anaesthesias before, even with MH inducing agents[26]. However, they may develop an MH crisis during an emergency operation and quite rarely during a pre-planned surgical procedure. The reason for this variability of triggering an MH crisis is still an enigma.

2.3.1. Sympathetic nervous system

A factor contributing to the triggering of MH is thought to be "stress", either emotionally or physically induced, or by elevated temperature. Thus, some kind of activation of the sympathetic nervous system may potentiate the triggering agents. However, controversial results have emerged from pigs sympathetically blocked by total spinal anaesthesia[27] or by bretyllium tosylate , plus adrenalectomy[28]. Studies by Kerr et al[29] on MH susceptible pigs (Poland-China), given lumbar epidural analgesia with lidocaine (2.8 $\pm$ 0.4 mg/kg), showed no signs of rigidity in the blocked hind-limbs, but at the same time muscular rigidity in the unblocked fore-limbs. In pigs with incompletely blocked hind-limbs a delay was seen in onset of muscle rigidity, but MH ensued in all incompletely blocked pigs. As the extent of the epidural block was not mentioned, it is unknown whether the adrenals had been blocked or not.

In contrast to these results[29] Gronert et al[27] showed that total spinal anaesthesia did not prevent MH. In eight Poland-China pigs, given a total spinal anaesthesia with isobaric tetracaine 1.2-2.2 mg/kg, halothane 1% or halothane 1% plus succinylcholine 3 mg/kg, produced MH. This was verified by the observation of muscular rigidity and the evaluation of the metabolic changes in arterial blood, the increased lactate concentration and increased $P_a CO_2$, and increased body temperature.

The effect of pretreatment with the adrenergic blocking agent bretylium, combined with adrenalectomy have led to conflicting results[28]. Pietrain pigs (n=6) treated with bretylium and adrenalectomized, produced no signs of MH on exposure to halothane 1% for 10 minutes. However, treatment with bretylium alone or adrenalectomy alone did not prevent the development of MH[28].

The different conclusions on the role of sympathetic blockade on the prevention of an MH crisis is rather confusing. In a review, Gronert[4] was unable to account for the different outcome of the two basically identical studies. Later on it appeared that the "non-reactor" pigs in the epidural study[29] reacted immediately by muscular rigidity and symptoms of MH on mechanical stimulation, like needle puncture or incision[4]. Therefore, the basis for the statement of a protective effect of epidural anaesthesia is rather weak. Furthermore, the exposure to halothane 1% alone in the

bretylium [28] treated group could have been a too weak trigger.

2.3.2. Barbiturates, neuroleptics

Studies on the variation in onset of porcine malignant hyperthermia by Gronert et al [30] have shown a delay in onset of an MH crisis by prior administration of thiopental. A positive correlation was observed between the dose of thiopental and the delay in onset of MH symptoms [30]. A proper anaesthetic dose of barbiturates is apparently able to delay an MH response. This observation may account for the variability of MH in humans.
A similar attenuation of the hyperthermic response in pigs has been observed after pre-treatment with neuroleptics [31].

2.3.3. Non-depolarizing muscle relaxants

The administration of a non-depolarizing skeletal muscle relaxant (pancuronium) to MH susceptible pigs, prior to the administration of halothane, was able to delay the onset of MH [30]; no other drugs, like thiopental, had been used. It should be noted that pancuronium was not able to prevent MH. Halothane used in the "barnyard" challenge produced signs of MH within 2-3 min. Treatment with pancuronium (0.5 mg/kg) prior to exposure to halothane delayed the MH response by 30-60 min.
A similar effect has been observed after pre-treatment of MH susceptible pigs with another non-depolarizing skeletal muscle relaxant: metocurine iodide [32].

In line with the observations of a delay in onset of MH are the observations of Ahern et al[22] showing a delayed decrease of PCr and ATP and a delayed increase of lactate in skeletal muscle in situ of MH susceptible pigs exposed to non-depolarizing muscle relaxants[25,32] and/or barbiturates[23] or neuroleptics[24,31].

It was concluded that depolarization of the skeletal muscle is necessary for the development of an MH crisis.

Studies on skeletal muscle membranes by Thesleff et al provided evidence for the effect of anaesthetics on the sarcolemma[33]. These authors showed that a pentobarbital concentration of 0.05 mg/ml in the tissue bath completely abolished the depolarizing effect of acetylcholine on the post synaptic membrane in the end-plate region. Pentobarbital, 0.15-0.29 mg/ml, produced a "significant increase in the electrical threshold of the membrane, and at the same time reduced or abolished the action potential"[33]. No effect was seen on the resting membrane potential. So barbiturates render skeletal muscle membranes in vitro less susceptible to depolarization.

The results of the latter study[33] on the effects of anaesthetics on the skeletal muscle membranes support the conclusions by Gronert et al[30] on the involvement of the depolarization of the skeletal muscle membrane in the initiation of MH. The reduced excitability of skeletal muscle membranes by barbiturates or non-depolarizing relaxants is apparently

a crucial factor in the delay of onset of MH.

In agreement with these results are the observations of Gallant et al [34] on the effect of halothane on the resting membrane potential of pig sarcolemma. The latter concluded that halothane in MH susceptible pigs reduced this potential towards the depolarization threshold of the skeletal muscle membrane[34]. This effect was not observed in the control pigs. Dantrolene produced a partial reversal of the halothane induced depolarization [34].

The same authors studied the effect of K^+ induced depolarization on pig skeletal muscle: skeletal muscle, taken from the MH susceptible pigs, developed a contracture at lower concentration of K^+ than muscle taken from control animals [35]. As the K^+ induced depolarization is equivalent to the electrically induced depolarization, it was concluded that: "the depolarization needed to activate the contractile mechanism in MH susceptible muscle cells is less than normal", and "the MH triggering agent halothane reduces the depolarization needed to activate muscle even further" [35].

2.4 GENETICS OF MH

Malignant hyperthermia is considered a pharmacogenetic disorder. This means that only the administration of certain drugs will induce the MH reaction in the apparently healthy subject[4]. MH in humans is inherited as an autosomal-dominant disorder, with variable expression and reduced penetrance[4,36].
However, the screening methods of MH have still to be viewed with a great deal of scepticism. The most reliable screening test is the so called "in vitro muscle pharmacological contracture test"[4], but this test is not a suitable mass screening method.

Based on the frequency of MH during general anaesthesia it is estimated that the incidence will be about 1 in 15,000 in children and 1 in 50,000 to 1 in 100,000 in adults, based on a random distribution of the MH trait in the population[39].

Exact figures can not be given for the Netherlands, as MH is not registered in the Dutch medical record registration office (SMR, Utrecht). Based on incidental reports we noted in 1981 five cases; in two cases with fatal outcome. Both cases were previously healthy young adults. The incidence in the U.S.A., Canada, Australia and U.K. is higher than in other countries, maybe due to the more generous use of volatile anaesthetics. Furthermore, in immigration countries

a higher incidence might be present due to the restricted genetic pool and the large number descendants. That is probably why the first reports and extensive studies came from Australia and U.S.A..

Due to the fact that still confusion exists on the exact criteria to trace MH susceptibility[39], the genetic pattern has not been established still. Furthermore, owing to the elaborate procedure of the in vitro contracture test, only a restricted amount of suspects can be investigated. Its invasiveness prohibits the use in children. Due to these restrictions, conclusions can be drawn only with some reservation on the inheritance pattern of MH in humans. Besides the already mentioned autosomal inheritance pattern with reduced penetrance and variable expression, some other investigators have stated that MH may be inherited in a way controlled by more than one gene[40]. In one case, both parents of a patient appeared not to be susceptible to MH, based on the in vitro contracture test[40]; this may point to a recessive trait.

Genetic studies on the inheritance pattern of pigs, using the halothane inhalation screening test have led to the contrasting conclusions. Eikelenboom et al[41] stated that MH is inherited in Dutch landrace pigs as recessive with complete penetrance. Britt et al[42] used as a screening method the halothane inhalation test plus the in vitro

muscle contracture test with caffeine, or caffeine plus halothane. They concluded that in pigs five phenotypes could be differentiated, pointing to the role of more than one gene controlling susceptibility of MH[42]. The investigations by Williams et al[43] employed both the halothane inhalation test and the administration of succinylcholine and the measurement of metabolic rate. They concluded that MH in pigs is inherited by a single dominant gene or two dominant genes.

Concluding: based on the genetic pattern in pigs, confusion has only become greater in clarifying the pattern of inheritance in humans.

2.5 MH IN RELATION TO OTHER SYNDROMES

From the beginning of the practice of general anaesthesia, case reports have emerged on hyperthermic episodes of patients receiving general anaesthesia[44,45]. Besides the possibility of an infectious process or pyrogenic reaction to transfusion fluids, the hyperthermic reaction was considered at that time as a kind of "post-operative heatstroke". It was thought that this heatstroke was induced by poor airconditioning of the operation theatre, or a high relative humidity. A great number of similar symptoms are present in heatstroke and malignant hyperthermia.

2.5.1. Heatstroke

This syndrome starts with neurological symptoms: headache, vertigo, faintness, pains (especially abdominal), confusion and finally coma[46]. On physical examination the most marked finding is the elevated temperature (41.1 ^{o}C or higher, rectally, and a skin temperature of 43.3 ^{o}C or higher). The skin is hot and dry, blood pressure is unstable, in the advanced cases shock is present. Respiration is shallow and rapid.
In contrast to MH, skeletal muscle tone is flaccid. Tendon reflexes are diminished. Urine production is decreased due to shock and a renal shutdown caused by haemoglobin as a result of haemolysis and by myoglobin due to rhabdomyolysis.
The laboratory findings in heatstroke show electrolyte disturbances and in the final stage signs of intra-

vascular coagulation. Therapy of heatstroke is quite similar to MH, namely rapid cooling of the patient, treatment of shock and vasodilation.
Some case reports have presented evidence for the beneficial use of dantrolene sodium in treatment of heatstroke, non reacting favourably on standard treatment[47]. The effectiveness of dantrolene sodium may point to some common mechanism in heatstroke and MH. In MH calcium influx may be induced by anaesthetics; in heatstroke calcium influx may be present secondary to the damage to the cellular membranes.

2.5.2. Neuroleptic malignant syndrome

Another rare syndrome, showing similarities with MH is the neuroleptic malignant syndrome (NMS)[48,49,50]. This syndrome is manifest in psychiatric patients, taking anti-psychotic drugs, either for many months or a few days. The symptoms are quite similar to MH: temperature elevation, tachycardia, unstable bloodpressure and mental confusion leading to coma. The serological changes are most markedly the elevation of CK, LDH and serum glutamate-oxalacetate transaminase (SGOT) levels.
No marked electrolyte changes are present.
The syndrome is preceded by a slight temperature elevation, hypokinesia or akinesia, sometimes with muscular rigidity. The fullblown case shows at marked muscular rigidity, hyperthermia (41 ^{o}C or higher), stupor or coma. The concomitant disturbances in swallowing

and salivation may lead to aspiration pneumonitis, aggravating the temperature elevation. Furthermore, the severe disturbances of the autonomic nervous system is manifest by the labile cardio-vascular system, leading to tachycardia, low bloodpressure and sweating. The fullblown case may take 24-72 hours for complete development. The triggering agents belong to the butyrophenone drugs haloperidol, thioproperazine and flufenazine. This in contrast to the observations in MH susceptible pigs, in which MH could be delayed by previous administration of the butyrophenone droperidol[31].

Treatment of NMS consists of terminating the administration of triggering drugs, cooling of the patient and cardiovascular stabilization. Quite effective is the administration of dantrolene sodium[51,52]. After i.v. administration of 1.25-1.5 mg/kg BW, the elevated temperature was noted to disappear within 1-2 hours.
The aetiology of NMS is unclear. It may interfere with the central thermoregulatory mechanisms, as the neuroleptics inducing NMS have also potent antidopaminergic properties. Pre-operative administration of neuroleptics has resulted in MH like symptoms, and these have been diagnosed erroneously as MH[53,54].

To exclude a common aetiology of NMS and MH, an in vitro muscle contracture test has been performed on a muscle sample of a patient who recovered from NMS[55].

A normal contracture pattern was observed. This observation excludes a common aetiology of NMS and MH.

2.5.3. Anticholinergics

Other diseases producing hyperthermia, like infectious diseases and fever due to malignancies are out of the scope of this chapter. However, intoxication with anti-cholinergic drugs have to be taken into account, especially in anaesthesia of children. The administration of anti-cholinergics in children after a long overnight fasting and a surgical procedure by the end of the day schedule, may produce feverish reactions, especially during hot summer days. Also mental confusion may be present, due to a central cholinergic effect.

2.5.4. Cerebral disorders

Neurotrauma, cerebral tumors, epileptic fits and cerebral haemorrhage produce feverish reactions. Depending on the extent and localization of the focus, concomitant neurological disturbances will be present, like stupor or coma.

2.5.5. Endocrine disorders

Endocrine disorders may also produce feverish reactions. Hyperthyroidism, unrecognised and so untreated prior to surgery mimics symptoms of MH. The occurrence of tachycardia and a dysregulation of the autonomic nervous system will lead to unstable bloodpressure and finally to shock. Tachycardia in hyperthyroidism can effectively be antagonised

with beta-blocking agents. This in contrast to tachycardia in MH, which does not react to the administration of beta-blocking agents.

2.5.6. Sudden infant death syndrome

It has been reported by Denborough et al[56] that a high incidence of MH susceptibility is observed in parents having lost a child due to sudden infant death syndrome (SIDS). It was noted that five of the fifteen parents tested, had an abnormal in vitro muscle contracture test on halothane 3% and on caffeine.

It has even been stated that SIDS may be comparable to heatstroke, since it was noted that the infants dying from SIDS were found to have elevated body temperature[57], just before death or immediatly after death. However, these statements have to be viewed with scepticism, as a great variety of other possible disorders have been related to SIDS.

Literature :

1. Denborough M A, Lovell R R H. Anaesthetic deaths in a family. Lancet 1960: ii-45.
2. Harrison G, Saunders S J, Biebuyck J F, Hickman R, Dent D M, Weaver V, Terblanche J. Anaesthetic induced malignant hyperpyrexias and a method for its prediction. Br J Anaesth 1969: 41, 844-855.
3. Britt B A, Kalow W. Malignant hyperthermia: a statistical review. Can Anaesth Soc J 1970: 17, 293-315.
4. Gronert G. Malignant hyperthermia. Anesthesiology 1980: 53, 395-423.
5. Caseby N G. Muscle hypertonus after intravenous suxamethonium: a clinical problem. Br J Anaesth 1975: 47, 1101-1106.
6. Crocker B D, Okumura F, McCuag D I, Denborough M A. Temperature monitoring during general anaesthesia. Br J Anaesth 1980: 52, 1223-1229.
7. Steward D J. Malignant hyperthermia: the acute crisis. In: Malignant hyperthermia. Britt B A, ed. Int Anesthes Clin 1979: 17, no. 4: 1-9.
8. Lucke J N, Hall G M, Lister D. Porcine malignant hyperthermia. I: metabolic and physiological changes. Br J Anaesth 1976: 48, 297-304.
9. Gronert G A, Theye R A. Halothane induced porcine malignant hyperthermia: metabolic and haemodynamic changes. Anesthesiology 1976: 44, 36-43.
10. Berman M C, Harrison G G, Bull A B, Kench J E.

Changes underlying halothane induced malignant hyperpyrexia in landrace pigs. Nature 1970: 225, 653-655.

11. Berman M C, Kench J E. Biochemical features of malignant hyperthermia in landrace pigs. In: International symposium on malignant hyperthermia, Gordon R A, Britt B A, Kalow W eds. Springfield: C C Thomas, 1973: 287-297.
12. Hall G M, Lucke J N. Porcine malignant hyperthermia: IX: changes in the concentrations of intramuscular high-energy phosphates, glycogen and glycolytic intermediated. Br J Anaesth 1983: 55, 635-640.
13. Isaacs H, Heffron J J A. Morphological and biochemical defects in muscles of human carriers of the malignant hyperthermia syndrome. Br J Anaesth 1975: 47, 475-481.
14. Mitchell G, Heffron J J A, Van Rensburg A J J. A halothane induced biochemical defect in muscle of normal and malignant hyperthermia susceptible landrace pigs. Anaesth Analg 1980: 59, 250-256.
15. Williams C H, Houchins C, Schanklin M D. Pigs susceptibility to energy metabolism in the fulminant hyperthermia syndrome. Br Med J 1975: 3, 411-413.
16. Gronert G A, Heffron J J A, Milde J H, Theye A. Porcine malignant hyperthermia: a role of skeletal muscle in increased oxygen consumption. Can Anaesth Soc J 1977: 24, 103-109.
17. Hall G M, Bendall J R, Lucke J N, Lister D. Porcine

malignant hyperthermia. II: heat production. Br J Anaesth 1976: 48, 305-308.

18. Liebenschütz F, Mai C, Pickerodt V M A. Increased carbon dioxide production in two patients with malignant hyperpyrexia and its control by dantrolene. Br J Anaesth 1979: 51, 899-903.
19. Hall G M, Lucke J N, Lovell R, Lister D. Porcine malignant hyperthermia. VII: Hepatic metabolism. Br J Anaesth 1980: 52, 11-17.
20. Hall G M, Lucke J N, Orchard C, Lovell R, Lister D. Porcine malignant hyperthermia. VIII: Leg metabolism. Br J Anaesth 1982: 54, 941-947.
21. McLoughlin J V, Mothersill C. Halothane induced rigidity and associated glycolytic and energy phosphate changes in red and white fibres of skeletal muscle of the pig. J Comp Path 1976: 86, 465-476.
22. Ahern C P, Somers C J, Wilson P, McLoughlin J V. Halothane induced malignant hyperthermia: creatine phosphate concentration in skeletal muscle as an early indicator of the syndrome. J Comp Path 1980: 90, 177-186.
23. Somers C J, Wilson P, Ahern C P, McLoughlin J V. Energy phosphate turnover and glycolysis in skeletal muscle of the pietrain pig: the effect of premedication with azaperone and pentobarbitone anaesthesia. J Comp Path 1977: 87, 177-183.
24. McLoughlin J V, Heffron J J A. The effect of aza-

perone on post-mortem changes in pig and rabbit skeletal muscle. Br Vet J 1975: 131, 102-107.

25. McLoughlin J V. Curare and post-mortem changes in skeletal muscle of pietrain pigs. Proc R I A 1974: 74B, 305-312.
26. Halsall P J, Cain P A, Ellis F R. Retrospective analysis of anaesthetics received by patients before susceptibility to malignant hyperpyrexia was recognized. Br J Anaesth 1979: 51, 949-954.
27. Gronert G A, Milde J H, Theye R A. Role of sympathetic activity in porcine malignant hyperthermia. Anesthesiology 1977: 47, 411-415.
28. Lucke J N, Denny H, Hall G M, Lovell G M, Lister D. Porcine malignant hyperthermia VI: the effects of bilateral adrenalectomy and pretreatment with bretylium on the halothane induced response. Br J Anaest 1978: 50, 241-245.
29. Kerr D D, Wingard D W, Gatz E E. Prevention of porcine malignant hyperthermia by epidural block. Anesthesiology 1975: 42, 307-311.
30. Gronert G A, Milde J H. Variations in onset of porcine malignant hyperthermia. Anesth Analg 1980: 60, 499-503.
31. McGrath C J, Rempel W E, Addis P B, Crimi A J. Acepromazine and droperidol inhibition of halothane induced malignant hyperthermia (porcine stress syndrome) in swine. Am J Vet Res 1981: 42, 195-198.
32. Roberts J T, Williams C H, Hoech G P et al. Prevention of halothane induced porcine malignant hyperthermia by

pretreatment with metocurine iodide. Anesthesiology 1982: 57, A 224.

33. Thesleff S. The effect of anesthetic agents on skeletal muscle membrane. Acta Physiol Scand 1956: 37, 335-349.
34. Gallant E M, Godt R E, Gronert G A. Role of plasma membrane defect of skeletal muscle in malignant hyperthermia. Muscle and nerve 1979: 2, 491-494.
35. Gallant E M, Gronert G A, Taylor S R. Cellular membrane potentials and contractile threshold in mammalian skeletal muscle susceptible to malignant hyperthermia. Neurosci Lett 1982: 28, 181-186.
36. Britt B A, Locher W G, Kalow W. Hereditary aspects of malignant hyperthermia. Can Anaesth Soc J 1969: 16, 89-98.
37. Kalow W, Britt B A, Richter A. The caffeine test of isolated human muscle in relation to malignant hyperthermia. Can Anesth Soc J 1977: 24, 678-694.
38. Rosenberg H, Reed S. In vitro contracture tests for susceptibility to malignant hyperthermia. Anesth Analg 1983: 62, 415-420.
39. Nelson T E, Flewellen F H. Current concepts, the malignant hyperthermia syndrome. New Eng J Med 1983: 309, 416-418.
40. Ellis F R, Cain P A, Harriman D G F. Multifactorial inheritance of malignant hyperthermia susceptibility. In: Malignant hyperthermia, second internationa symposium, Aldrete J A, Britt B A, eds. New York: Grune & Stratton 1978: 329-338.

41. Eikelenboom G, Minkema D, Van Eldik P, Sijbesma W. Inheritance of the malignant hyperthermia syndrome in Dutch landrace swine. In: Malignant hyperthermia, second international symposium, Aldrete J A, Britt B A, eds. New York: Grune & Stratton 1978: 141-146.
42. Britt B A, Kalow W, Endrenyi L. Malignant hyperthermia pattern of inheritance in swine. In: Malignant hyperthermia, second international symposium, Aldrete J A, Britt B A, eds. New York: Grune & Stratton 1978: 195-211.
43. Williams C H, Shanklin M D, Hedrik H B, Muhrer M E, Stubbs D H, Krause G F, Payne C G, Bendict J D, Hutcheson D P, Lasley J F. The fulminant hyperthermia stress syndrome: genetic aspects, hemodynamic and metabolic measurements in susceptible and normal pigs. In: Malignant hyperthermia, second international symposium, Aldrete J A, Britt B A, eds. New York: Grune & Stratton 1978: 113-140.
44. Tuttle J P. Heatstroke as a post-operative complication. J Am Med Ass 1900: 35, 1685.
45. Moschocowitz A V. Heatstroke as a post-operative complication J Am Med Ass 1900: 35, 1685.
46. Petersdorf R G. Alterations in body temperature. In: Harrison´s principles of internal medicine, Wintrobe M W et al eds, 7th edition, Tokyo: McGraw-Hill 1974: 48-62.

47. Lydiatt J S, Hill G E. Treatment of heatstroke with dantrolene. J Am Med Ass 1981: 246, 41-42.

48. Geller B, Greydanus D E. Haloperidol induced comatose state with hyperthermia and rigidity in adolescence: two cases reports with a literature review. J Clin Psychiatry 1979: 40, 102-103.

49. Caroff S N. The neuroleptic malignant syndrome. J Clin Psychiatry 1980: 41, 79-83.

50. Van Dijke C P H. Het maligne neuroleptica syndroom. Geneesmiddelenbulletin 1983: 17, 7-9.

51. Goekoop J G, Carbaat P A Th. Treatment of neuroleptic malignant syndrome with dantrolene. Lancet 1982: ii, 49-50.

52. Coons D J, Hillman F J, Marshall R W. Treatment of neuroleptic malignant syndrome with dantrolene sodium: a case report. Am J Psychiatry 1982: 139, 944-945.

53. Moyes D. Malignant hyperpyrexia caused by trimeprazine, Br J Anaesth 1973: 45, 1163-1164.

54. Haberman M. Malignant hyperthermia: an allergic reaction to thioridazine. Arch Int Med 1978: 138, 800-801.

55. Tollefson G. A case of neuroleptic malignant syndrome: in vitro muscle comparison with malignant hyperthermia. J Clin Pharmacol 1982: 2, 266-270.

56. Denborough M A, Galloway G J, Hopkinson K C. Malignant hyperthermia and sudden infant death. Lancet 1982: ii, 1068-1069.

57. Stanton A N, Scott D J, Downham M A P S. Is overheating a factor in some unexpected infant deaths? Lancet 1980: i, 1054-1057.

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Pathophysiology and aetiology of malignant hyperthermia (MH)

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PATHOPHYSIOLOGY AND AETIOLOGY OF MALIGNANT HYPERTHERMIA :

3.0 Introduction and restriction

Considerable experimental work has been performed in order to elucidate the pathophysiological mechanism(s) and the aetiology of malignant hyperthermia (MH). Most of this work has been done on the animal model of MH, i.e. in pigs. It may be questioned whether this model can be extrapolated to the human situation. MH in pigs can be initiated by stress, by the administration of volatile anaesthetics and/or by succinylcholine. The volatile anaesthetics and/or succinylcholine initiate MH in humans. Furthermore, a single case report claims the triggering of MH in humans due to stress. The biochemical changes during an MH crisis in humans and pigs are identical. Increased oxygen consumption, increased CO_2 output, increased lactate production in skeletal muscle and a generalized acidosis are common features, besides muscle rigidity and increased heat production. Studies on the porcine model have to be performed using proper stress reduction, since stress alone may induce MH without any exposure to triggering agents.

With respect to the triggering agents, a parallel may be drawn between the porcine MH model and MH in humans.

Data on the effects of triggering agents on isolated

skeletal muscle or isolated organelles have to be extrapolated with caution to the intact animal, as it is still unknown which factor causes the initiation of MH. It is hypothesized that an increased myoplasmic influx of calcium occurs during an MH crisis. This increased amount of calcium will initiate muscular rigidity, metabolic disturbances and heat production. The origin of this calcium influx has not been elucidated yet.

In intact skeletal muscle considerable differences exist in calcium concentrations at rest: extracellular ion concentration is about 10^{-3} M, representing 50% of the total calcium molecules of the extracellular fluid. The intracellular concentration of calcium is about 10^{-7} M, i.e. a calcium gradient exists of about 10,000. During excitation intracellular calcium increases to about $2{:}10^{-4}$M. Most of this calcium is released from sarcoplasmic reticulum (SR). At rest, a concentration gradient of about 2,000 exists between the sarcoplasma and the sarcoplasmic reticulum (SR). This gradient is built up in an energy dependent way.

Studying isolated skeletal muscle organelles it is clear that considerable damage will occur to the mechanisms regulating intracellular calcium levels, as well as to the anatomical situation.
The results of experiments on isolated organelles or skeletal muscle may be helpful, but are artificial.

3.1 Central Nervous System and MH

The role of the central nervous system in MH has been debated for a long time[1]. At first MH was considered to be of central origin: i.e. a disturbance of the thermoregulatory control system. However, studies on the porcine MH model have shown that skeletal muscle is the target organ in the MH syndrome[1].

Before the recognition of MH as a syndrome, it was assumed that, due to the action of general anaesthetics, - especially the volatile anaesthetics -, a disturbance occurred of the thermoregulatory centre of the CNS. Furthermore, MH would possibly be induced by environmental circumstances: high relative humidity, draping of the patient which prevented heat dissipation, and a poor ventilation of the operation theatre. In this respect, MH was considered to be a kind of a post-operative heat stroke.

Studies on cerebral metabolism during porcine malignant hyperthermia have led to the following results :

1. Cerebral oxygen consumption is not increased during the MH crisis, in contrast to the total body oxygen consumption, which is increased 2-3 times.
2. No increase is measured of the cerebral venous concentration of K^+ or lactate.

From these findings it was concluded that the central nervous system is not primarily involved in the

pathogenesis of an MH crisis.

The observed neurological changes in humans, like coma, are due to the severe hypoxia and the high temperature, and severe acidosis, which are known to produce severe damage to the central nervous system.

3.2 Neuro-humoral changes in MH

Due to the role of stress in the initiation of MH in pigs, a number of investigations have been devoted to the role of the autonomous nervous system in MH [3]. Stress reducing drugs belonging to the butyrophenone group (droperidol, azaperone), and barbiturates are known to prevent stress induced MH in pigs and to attenuate MH, induced by anaesthetics [3].
During an MH crisis increased catecholamine levels are found in plasma of pigs [4]. Whether this is a primary reaction or secondary to the MH reaction is open to debate. During an MH period, increased levels of noradrenaline, adrenaline, glucagon and cortisol are present, both in humans and in pigs [5].

Studies on the effects of adrenergic blocking agents have led to inconclusive results. Pigs pretreated with bretylium tosylate (an adrenergic blocking agent) and bilateral adrenalectomy, did not show an MH reaction to halothane [6]. Bilateral adrenalectomy or treatment with bretylium alone (10 or 20 mg/kg BW) did not have protective effects against a halothane challenge [6]. The infusion of the alpha adrenergic blocking agent phentolamine at very high doses (50 µg/kg BW) prevented a halothane induced challenge, whereas at lower concentrations no protection was seen. The infusion of beta blocking agents (propranolol) did not offer any protection.

Complete sympathetic blockade by total spinal anaesthesia in pigs[7] showed no protection against a halothane challenge. Therefore, the observed increased levels of catecholamines during an MH crisis are secondary to the metabolic changes during an MH crisis. It is still an unresolved matter why MH can be triggered by stress in MH susceptible pigs.

Gronert et al[8] observed that in an isolated perfused hind-limb, carbachol (an acetylcholine derivate) induced symptoms of MH (muscular rigidity, increased lactate production and increased oxygen consumption). In control pigs carbachol did not produce signs or symptoms of MH. A common effect of carbachol and alpha-adrenergic stimulators is the rapid hydrolysis of phosphatidylinositol on the cellular membrane, producing cyclic phosphate and diacylglycerol[9].
In the presence of calcium, diacylglycerol activates protein kinase-C. This enzyme is involved in protein phosphorylation, a major mechanism controlling intracellular processes[9].

The role of the second messenger calmodulin in the aetiology of MH is still under study. Calmodulin is activated by an increased intracellular calcium concentration[10,11,12,13]. The activated calmodulin initiates three major processes in skeletal muscle[11]: 1: it activates phosphorylase kinase, converting the inactive phosphorylase-b into the active phosphorylase-a, leading to glucogenolysis and inhibition of glycogen synthesis. 2: it activates myosine-light-chain-kinase (MLCK) phosphorylating myosine, (which interacts with actine) and so produces

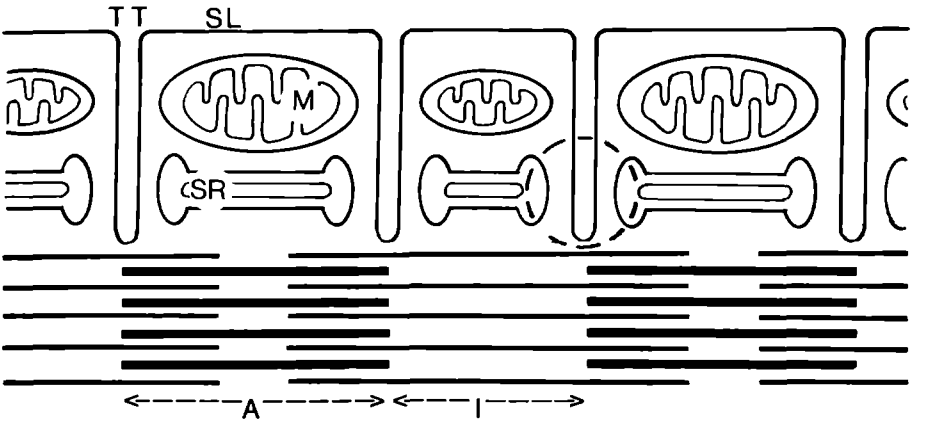
muscular contraction. 3: in skeletal muscle it interacts with the sarcoplasmic protein-phosphorylating system, regulating the calcium release from the SR during excitation.

The interrelation between cyclic-AMP and calmodulin is a complex one. Both behave as a second messenger: cyclic-AMP levels increase after beta-stimulation and calmodulin levels increase after electrical excitation. Both second messengers have the same effects on metabolism in skeletal muscle: they produce glycogenolysis and phosphorylation of proteins in the sarcoplasma [8,11].
Gel-electrophoretic studies on possible abnormalities in calcium binding proteins in MH susceptible pigs did not reveal any differences in calmodulin obtained from MH susceptible pigs compared to control pigs [14].

3.3 Muscular system

A considerable number of investigations have been carried out on skeletal muscle or organelles of skeletal muscle. This has been done in order to find a possible aetiological factor in the pathogenesis of MH. Most of these studies had to be performed on skeletal muscle obtained from pigs. We will discuss the components of skeletal muscle and the effect of MH inducing drugs on these components. For a scheme of anatomy see figure 1.

Figure 1. Schematic drawing of skeletal muscle in man. Illustrated organelles and anatomical interrelations are not in scale.



A = A-band
I = I-band
M = mitochondrion
SR = sarcoplasmic reticulum
SL = sarcolemma
TT = transverse tubule
= triad

3.3.1 Sarcolemma

The role of the sarcolemma in the aetiology of MH has been investigated by several groups. Halothane 2% produced a depolarizing of the resting membrane potential by about 5-15 mV in MH susceptible pigs, this effect on the membrane potential was not seen in muscle samples taken from control pigs[15]. Before exposure to halothane, no difference was seen between the resting membrane potentials of the MH susceptible pigs and the controls. The addition of dantrolene reduced the induced depolarization by

halothane in MH susceptible muscle, and was maximal at a concentration of 40 μM.

The mechanical threshold (the membrane potential at which a microscopically visible threshold-contraction is produced) of skeletal muscle of MH susceptible pigs is lower than in control pigs. A depolarization of about 10 mV causes a contraction[17].

The increased sensitivity of muscle obtained from MH susceptible pigs to external K^+ is in line with this observation. The MH susceptible skeletal muscle responded at a lower concentration of K^+ with a higher contractile response, as compared to the control muscle samples[17]. Addition of halothane produced a further shift to the left of this dose response curve. So the level of depolarization needed to activate the contractile mechanism in MH muscle cells, is less than normal and halothane reduces the depolarization, needed to activate the muscle. It was concluded that the sarcolemma in MH susceptible pigs is involved in the triggering of an MH reaction.

3.3.2 Tubular system

The tubular (T) system forms the anatomical and functional connection between the sarcolemma and the interior of the muscle cell[18,19]. In mammalian skeletal muscle the T-system is an invagination of the sarcolemma and runs perpendicularly to the longitudinal axis of the skeletal muscle cell. The junction of the T-system with the SR is called a "triad". This triad is composed of 2 terminal cisternae of the SR and 2 T-tubules. These triads are situated at the level of the A-I boundary (see figure 1).

Activation of the contractile system is initiated by a depolarization of the sarcolemma. This depolarization is propagated by the T-system into the interior of the skeletal muscle fibre. The depolarization of the T-system results in the release of calcium ions out of the terminal cisternae of the adjacent SR. Upon depolarization of the T-system a small amount of Ca^{2+} is entering the sarcoplasma at the triads. This small increase of Ca^{2+} leads to the release of a higher amount of Ca^{2+} from the SR, sufficient to produce a muscular contraction. This is the so called "Ca^{2+} induced Ca^{2+} release"[18,20].

Maximal activation of the contractile system is achieved at a concentration of Ca^{2+} of 10^{-6} Mol in the bathing solution of skinned muscle fibres. At 10^{-7} Mol a contraction is initiated. This is also the

Ca^{2+} concentration at rest in the sarcoplasma. Full excitation of the T-system and the SR produces a Ca^{2+} concentration of 2×10^{-4} M in the sarcoplasma.

The role of the T-system in MH is still not clear. As it was postulated that a depolarization of the sarcolemma is needed to trigger an MH crisis, further studies on the mechanism of excitation contraction coupling have been performed. By using special preparation techniques it is possible to remove the sarcolemma chemically[21]. The T-system can be disrupted selectively, using glycerol[21]. Treatment of the muscle fibres with D_2O (deuterium oxide) leads to the inhibition of the transmission of the depolarization from the T-system to the SR[22]. Using these various techniques on skeletal muscle taken from MH susceptible pigs, it is possible to reveal some aspects of the T-system in the triggering of MH. Studies by Okumura et al[23] have led to the conclusion that D_2O is able to abolish completely all chemically induced contractures by caffeine, halothane, suxamethonium, potassium chloride and thymol. This means that the depolarization of the T-system is essential for the development of a contracture. Glycerinating of the muscle fibres abolishes the electrically induced contractions, and reduces significantly the caffeine induced contracture[23]. A complete disappearence of the induced contracture by succinylcholine, halothane 3%, thymol 100 µMol and KCl 80 mMol is observed. These results support the

hypothesis of a defect of either the sarcolemma or the T-system.

On the other hand, the observation of the potentiating effect of halothane on the caffeine induced contracture of the glycerinated muscle points to a dual effect of halothane on skeletal muscle [24]. After disruption of the T-system the direct effect of halothane on the initiation of a contracture is abolished. This means that depolarization is a major factor in the triggering of MH. Furthermore, the caffeine induced contracture is still potentiated by halothane in the glycerinated muscle. This means that halothane is acting also beyond the level of the T-system in the excitation contraction coupling. It is thought that halothane enhances the release of Ca^{2+} out of the SR.

The role of the T-system in the triggering of MH is also illustrated by the effect of dantrolene sodium. Studies of the effects of dantrolene sodium on skeletal muscle specimens have shown that dantrolene has an inhibitory effect on the coupling between the T-system and the SR [25], besides an inhibitory effect on the release of Ca^{2+} from the SR [25].

3.3.3 Sarcoplasmic Reticulum

The sarcoplasmic reticulum (SR) is the organelle in the skeletal muscle mainly involved in the regulation of intra-cellular calcium levels during muscular contraction and relaxation[19]. In the resting state the intracellular calcium concentration is about 10^{-7} M, during muscular contraction it is about 2×10^{-4} M. The origin of this calcium is the SR and the increased calcium level lasts about 30 msec. In this period muscular contraction occurs. The uptake of the intracellular calcium in the SR is ATP dependent. The uptake of two calcium ions is accompanied by the hydrolysis of one molecule of ATP. The uptake follows a saturation kinetics with a K_M of 5.10^{-7} M Ca^{2+}. The total uptake capacity of calcium in the SR depends on the presence of Pi or oxalate in the incubation medium. If one of these components is present, the total uptake capacity is increased[26]. The total uptake capacity of SR in the absence of oxalate or Pi is about 2.10^{-7} M Ca^{2+}/mg protein[27]; in the presence of either 5 mM oxalate or 5 mM Pi the uptake capacity is about $7.5 .10^{-7}$ M Ca^{2+}/ mg protein[4]. This difference is due to the precipitation of insoluble Ca^{2+} salts with either oxalate or Pi in the SR. When these precipitations have been formed, the release of calcium out of the SR is reduced by a factor of ten[28]. Therefore, studying the kinetics of calcium uptake and release, it is important to define the exact incubation conditions.

In view of the hypothesis that an increased concentration of calcium is the main cause for the observed symptoms of MH as well as its effect on mitochondrial functioning (see 3.3.4.3), a considerable number of investigations has been performed on the calcium accumulation and release by the SR. Contradictory results have been reported [1,29].

At present, three different procedures of studying calcium kinetics exist.

1. Calcium binding (oxalate absent);
2. Calcium uptake velocity and capacity (oxalate present);
3. The spontaneous release of calcium.

The difference between the calcium binding studies and the uptake studies is based on the fact that in the absence of oxalate, binding of calcium is inhibited due to saturation of the affinity locus of the transport protein for calcium. The uptake studies in the presence of oxalate are considered to represent the physiological conditions of the intact cell, and therefore are considered to represent uptake in vivo [29].

Uptake and binding of calcium in SR obtained from the m.semitendinosus of MH susceptible pigs and controls, are shown in table 1. The kinetics of calcium uptake in the SR and the influence of halothane, at 37 ^{o} C, pH 6.9 are summarized.

Table 1 :

Calcium binding and uptake in sacoplasmic reticulum from control pigs and malignant hyperthermia susceptible (MH) pigs. Values are expressed as mean ± SEM, * significant difference at $p < 0.05$.

	Control pigs (n=5)	MH pigs (n=5)
Ca^{2+} binding: (10^{-9} moles Ca^{2+}/mg protein/min)	145 ± 10	105 ± 10
Ca^{2+} uptake rate: (10^{-9} moles Ca^{2+}/mg protein/min)	2.01 ± 0.11	1.29 ± 0.12 *
Ca^{2+} uptake capacity: (10^{-6} moles/mg protein)	3.29 ± 0.18	2.20 ± 0.17 *

From: Gronert G A et al, Eur J Pharmacol 1979: 58, 179-187 (ref. 29).

The yield of SR protein per gram of skeletal muscle was in both types of pigs about 3.0 mg/g skeletal muscle. A pre-existently lower uptake capacity and uptake rate was observed in SR obtained from MH susceptible pigs compared to controls. On exposure to halothane, no significant differences were measured in the calcium uptake rate and uptake capacity of SR in both types of pigs.

The addition of halothane to calcium loaded SR produced an increased release of calcium in a dose related way to the halothane concentration. No significant differences in release of calcium was observed between SR obtained from MH susceptible pigs and controls.

These data on calcium uptake and release were found to produce insufficient differences accounting for the observed

physiological and biochemical changes of the MH crisis[29].

3.3.3.1. Effects of pH and temperature on SR

Changes in pH and temperature may contribute to the further disturbance of calcium homeostasis in the sarcoplasma when the MH crisis has already been triggered. The effects of these changes have been studied by Berman et al[30] and by Nelson et al[31]. These workers concluded that a decrease of pH below 6.4 inhibits the uptake of calcium, and at pH values below 5.8 abolished it completely. The ATP-ase activity, however, is increased by 75% in the pH range 5.6 - 6.2. So, on decreasing pH, both calcium accumulation in SR is inhibited and increased hydrolysis of ATP occurs. It is known from our own observations in the MH pig model[32], that already after a 10 minutes´ challenge a significant increase of lactate in muscle occurs. So a "favourable" situation exists for the decreased uptake of calcium in the SR and an increased breakdown of ATP.

On increasing temperature, the SR from MH susceptible pigs have a lower calcium binding capacity than SR taken from control pigs.[31]
The difference between the MH susceptible pigs and controls in binding capacity at 39 ^{o}C is about 40 nmoles Ca^{2+}/mg protein. This difference in calcium binding capacity, is too low to account for an increased calcium concentration in the sarcoplasma to explain the pathogenesis of MH.

3.3.3.2. Pharmacological effects on SR

The in vitro screening test to evaluate MH susceptibility is based on the increased sensitivity to caffeine and/or halothane of skeletal muscle obtained from MH susceptible subjects [33]. The observed rigidity, evoked by exposure to caffeine and the observed rigidity during an MH period, have led to a theory of a common underlying mechanism in pathophysiology of MH comparable to the action of caffeine on skeletal muscle.

Caffeine acts on skeletal muscle by a potentiation of the calcium induced calcium release from the SR [34]. The concentration of calcium needed to induce calcium release from the SR at physiological conditions is about 10^{-4} M Ca^{2+}.[34]
The loading level of the SR with calcium influences the calcium induced calcium release from the SR [35]. Lightly loaded SR does not release calcium on exposure to calcium, on the contrary it will accumulate calcium. Caffeine shifts the dose-response curve for the calcium induced calcium release to the left. In the presence of 2 mM caffeine, 10^{-6} M Ca^{2+} induces release of calcium from the SR.

It has been claimed recently [36], that in skeletal muscle of a human susceptible to MH, the calcium induced calcium release occurred at significantly lower concentrations of calcium as compared to control individuals. Halothane

potentiated the calcium induced calcium release to the same extent, in muscle fibres both of the MH susceptible subjects and controls[36]. However, this is an isolated finding on SR obtained from only one MH susceptible subject.

A number of studies has been conducted to elucidate the interaction of other drugs with the caffeine induced calcium release from the SR. As stated before, a parallel can be drawn between the effect of caffeine on the SR and the aetiology of MH[38]. Therefore, interference with the caffeine induced effects on the SR is a rational basis for the treatment of MH. Initially, it had been claimed that the ester-type local anaesthetics were the drugs of choice in treating MH, especially procaine. This was based on the inhibitory effects of procaine on the caffeine induced increase of muscular tension in the porcine model[37]. At pH 7.4 procaine 5 mM inhibits the contracture produced by caffeine 1 mM plus halothane 1% in vitro. Lidocaine 5 mM (an amide type local anaesthetic) did not prevent the induced contracture. The concentrations of procaine and lidocaine used, were far in the toxic range for humans. Therefore, these observations have to be extrapolated to the human situation with caution. Furthermore, procaine did not reduce an existing contracture. On addition of procaine 2 mM to a muscle exposed to caffeine 0.75 mM plus halothane 1%, still a contracture occurred.

These results seem to justify some reservations regarding the appropriateness of procaine in the treatment of MH.

After the initially claimed therapeutic effect, it was shown later on in the MH pig model as well as in the treatment of MH crisis in humans, that procaine is not the first therapeutic agent of choice to treat MH. It is still claimed to be the drug of choice in treating cardiac arrythmias during an MH crisis in humans[1], based on the aggravating effect of lidocaine on the caffeine plus halothane induced contracture in vitro[37].

3.3.4.0 Mitochondrial function in relation to MH

Mitochondrial function can be evaluated in vitro, using the mitochondrial fraction of homogenized tissue. This method of studying mitochondrial function does not allow to extend the results straightforward to the intact animal or human subject[38]. This is due to the unphysiological medium in which the isolated mitochondria are suspended. Furthermore, many biochemical studies are performed at unphysiological temperatures, namely 25 ^{o}C instead of the normal body temperature.

Mitochondrial function in vitro is measured by oxygen consumption and phosphorylation of ADP to ATP; it only indicates a maximal capacity in vitro. In addition, under in vitro conditions, only the production of energy can be studied and the energy consumption of the in vivo situation is not taken into account.

When studying mitochondrial functioning in vitro, only those enzymes, involved in the oxicative phosphorylation, electron transport and oxidation of pyruvate, fatty acids and some amino acids, are considered[39]. Since all glycolytic enzymes are situated in the cytoplasm, no activity is measured of the glycolytic enzymes.[39]

Considering these facts, it is of utmost importance to define the experimental conditicns, especially

the substrates used. It is known from studies by Miller et al[40] and Cohen[38] that halothane inhibits the oxydation of the substrate glutamate and malate, but not the oxydation of the substrate succinate, all in the presence of oxygen and of a phosphate acceptor, ADP. For a better understanding of mitochondrial functioning, two parameters should be elucidated, namely the P/O ratio and the respiratory control index.

A. P/O Ratio :

Schematically[2]:

Electrons are provided by the Krebs cycle, when acetyl-CoA is degraded after the oxydation of carbohydrates, fatty acids or amino acids. In the Krebs cycle, acetyl CoA is degraded into 2 molecules of CO_2 and 4 pairs of hydrogen atoms. From the latter, the corresponding electrons are supplied to the electron transport chain.
In the last step of the electron transport chain, the electrons react with oxygen. During this electron transport process along the electron transport chain, phosphorylation occurs of ADP into ATP (oxydative phosphorylation). Depending on the substrate used, either 3 or 2 molecules of ATP are formed per atom oxygen consumed. This is the so called P/O ratio (the number of Pi taken up to phosphorylate ADP). The P/O ratio is three for the glutamate related substrates (pyruvate,

isocitrate, alphaketoglutarate and malate). The P/O ratio for succinate is two, as well as for the substrates fatty acyl Co-A and glycerol phosphate, because the first step of the electron transport chain is bypassed by these substrates.

This first step is the electron transport location between NADH and coenzyme Q. In this step, 1 molecule of Pi is phosphorylated with ADP into ATP. This is the very step, which is inhibited by general anaesthetics like halothane and barbiturates [38,40].

The other two locations in the respiratory chain, phosphorylating ADP into ATP, are unaffected by general anaesthetics [38,40].

These other two locations are the electron transfer steps between cytochrome b and -c and between cytochrome a_3 and oxygen.

B.Respiratory Control Index(Acceptor Control Index):

The Respiratory Control Index (RCI) is used as an index for the integrity of the mitochondria.

This index is expressed as the ratio between the so called state 4 and state 3 conditions. State 3 is the condition in which all necessary substrates are present for the oxydative phosphorylation.

State 4 is the condition in which the phosphate acceptor ADP is lacking. In state 3, the mitochondria are maximally stimulated. Knowing the amount of ADP added and the amount of oxygen consumed, the P/O

ratio can be calculated. State 4 is the condition when the phosphate acceptor ADP has been consumed completely during the state 3 condition, or has not been added to the incubation medium.

In almost intact mitochondria the RCI exceeds the value of ten. When the mitochondria are damaged, the RCI ratio is reduced, up to the lowest ratio of one. This means that the addition of a phosphate acceptor does not increase the oxygen consumption. So, the oxygen consumption in those conditions mentioned, is the same. The importance of the RCI is - besides of giving information on damage to the mitochondria - whether inhibition or "uncoupling" of the oxydative phosphorylation occurs.

Uncoupling means that oxygen consumption continues, but the phosphorylation of ADP does not occur. The P/O ratio becomes zero, and the RCI becomes one, as the oxygen consumption in state 3 and state 4 is maximally stimulated and remains unchanged. So, uncouplers produce a maximal oxygen consumption, without the production of ATP. It has been thought initially, that malignant hyperthermia might be a feature of uncoupling of oxydative phosphorylation.

Inhibitors of the oxydative phosphorylation prevent the phosphorylation of ADP to ATP and prevent the oxygen consumption by the addition of ADP. Consequently,

the mitochondria stay basically in state 4, both in the presence and the absence of ADP. The P/O ratio in state 4 remains unchanged, the P/O ratio of state 3 is not stimulated by the addition of ADP and remains unchanged, as compared to state 4. The RCI has a value of one, when an inhibitor is used, and the P/O ratio of state 3 is zero, because the added ADP is not phosphorylated to ATP.

3.3.4.1. The basis for the study of mitochondrial functioning

The basis for the usefulness of analysing mitochondrial functioning in MH is found in the increased oxygen consumption, increased carbon dioxide production and the increased body temperature during an MH crisis. An attractive hypothesis concerning these features mentioned is the uncoupling of oxydative phosphorylation[41]. This uncontrolled oxygen consumption during the MH crisis and the production of heat by the futile electron transport chain would be explained by the uncoupling hypothesis. A considerable number of experiments have been performed, in trying to prove the hypothesis of uncoupling of oxydative phosphorylation initiated by the anaesthetics, triggering MH.

Results of studies with porcine mitochondria by Brooks and Cassens[42], obtained from stress susceptible pigs and from control pigs, gave results as depicted in table 2. Experiments were done at 25 ^{o}C, 37 ^{o}C and 43 ^{o}C, no halothane

was used and the muscle samples were taken post-mortem from the m.longissimus dorsi (predominantly a white muscle) and from the m.trapezius (predominantly a red muscle).

Table 2 :

RCI and P/O ratio in mitochondria obtained from MH susceptible pigs and controls

	Control pigs (n=6)			MH pigs (n=6)		
	25 °C	37 °C	43 °C	25 °C	37 °C	43 °C
P/O ratio						
m.trapezius	2.3	2.2	2.1	2.5	2.4	2.2
m.longissimus	2.35	2.2	2.1	2.6	2.4	2.2
RCI						
m.trapezius	7.2	6.1	4.1	7.0	6.6	4.2
m.longissimus	5.8	5.2	4.2	5.2	5.1	3.5

From: Brooks G A et Al, J Anim Sci 1973: 37, 688-692 (ref. 42)

Increasing the temperature of the incubation medium produced an increase of the oxygen consumption, both in state 3 and state 4 and in the samples from MH suspectible pigs to the same extent as in the control samples. However, at 43 °C a decrease was measured in the P/O ratio.

Besides the decrease of the P/O ratio at 43 °C, a more important result is the decreased RCI. This means that the non-conservative respiration (state 4) is increased and so oxydative phosphorylation becomes less efficient

at this high temperature.

The effect of increasing temperature on oxydative phosphorylation is therefore not specific and does not add information on the aetiology of MH. The effect of the increased temperature on the oxydative phosporylation, contributes in the ultimate stage of MH to the decrease of energy-rich-compounds due to the decreased production capacity. Several other investigators [43,45], studying metabolism of porcine skeletal muscle mitochondria, have drawn the same conclusions as Brooks et al[42] on the oxydative phosphorylation as a possible aetiological factor in MH: no difference exists in oxydative phosphorylation capacity of skeletal muscle mitochondria when comparing MH susceptible pigs to control pigs. The effect of halothane on oxydative phosphorylation is a dose related inhibition of NADH dependent substrates. No difference is found in the effect of halothane on MH susceptible or control skeletal muscle mitochondria.

Information on mitochondrial function in MH susceptible humans is limited. The study by Britt et al[46] on 5 MH susceptible humans has led to the following conclusions : no difference exists in oxydative phosphorylation capacity in mitochondria of MH susceptible humans and control humans, both in the NADH dependent substrates (pyruvate, malate) and in the succinate dependent substrates. The addition of halothane caused the same dose related depression of oxydative phosphorylation of NADH dependent substrates.

No increase is measured of the state 4 oxygen consumption in the presence of halothane. So, halothane does not induce uncoupling of oxydative phosphorylation in skeletal muscle mitochondria of MH susceptible humans.

3.3.4.2. Effect of pH on oxydative phosphorylation

All previously mentioned mitochondrial investigations have been performed at a pH in the physiological range of the incubation medium. However, it has been shown that during an MH attack the pH of the skeletal muscle drops dramatically [1]. Also the venous blood, draining the hindlimbs in the porcine MH model, shows a decreased pH, due to an increased lactate content as well as an increased CO_2 content [1]. A study on the influence of the pH on oxydative phosphorylation capacity of mitochondria has been performed by Mitchelson and Hird [47]. A decrease of pH below 6.5 resulted in an inhibition of oxygen consumption in state 3, and a slight increase of oxygen consumption in state 4. This means that the P/O ratio at pH 60 drops to about 40% of the P/O ratio at pH 7.0 (substrate pyruvate plus malate).
The combined effect of halothane and pH on mitochondrial respiration is a potentiation of each others effect. At a pH lower than 6.0 halothane 1.2 mM (about 5% v/v) P/O ratio was reduced by 30% as compared to the P/O ratio at the same pH. The measurements of oxygen consumption during the combined studies on

the effects of halothane and pH show a pH- and halothane concentration dependent decrease. At a halothane concentration of 1.0 mM, oxygen consumption decreases from 90% of control value at pH 6.9 to 35% of control value at pH level of 5.7. This means that respiration is inhibited markedly and oxydative phosphorylation is only lightly uncoupled.

These observations of the combined effects of halothane and decreasing pH, are insufficient to explain the metabolic events of an MH crisis. No clue is added by these experiments on the aetiology of MH. These observations can only explain the secondary effects on metabolism of an MH crisis: the lowering pH during an MH attack may contribute to the decreased production of ATP by oxydative phosphorylation.
On the other hand, the decreased oxygen consumption in vitro of the mitochondria, exposed to increasing concentrations of halothane and decreasing pH values, is contradictory to the observed increased oxygen consumption during an MH crisis[1]. So, some other factors have to be involved in the triggering of MH and consequently the metabolic disturbances.

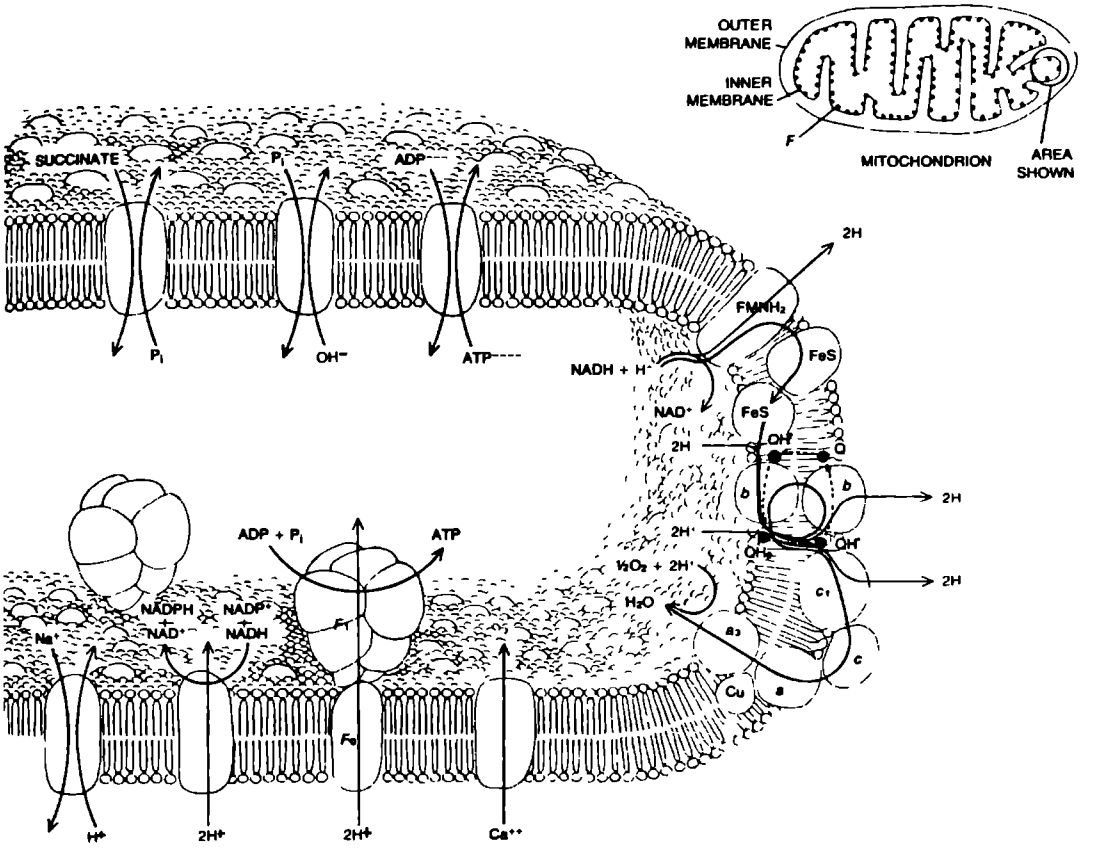
3.3.4.3 Calcium and mitochondrial functioning

It has been supposed that calcium is possibly the triggering agent of the MH crisis[1].
Calcium interferes with oxydative phosphorylation,

by interacting with the phosphorylation sites in the respiratory chain[39,48,49]. Studies have been performed on the influence of calcium on mitochondria in vitro, of MH susceptible pigs[44,45,50]. The transport of calcium into the mitochondria has been a subject of biochemical research. Generally its importance is due to its "second-messenger" behaviour in the cell[10,13]. Besides being involved in the excitation contraction coupling of skeletal or cardiac muscle, calcium is also involved in the regulation of intracellular biochemical processes[9,13].

In skeletal muscle cells, intracellular calcium concentration at rest is kept at a level of about 10^{-7} M[19]. An increase of intracellular calcium, e.g. during muscular contraction, is counteracted in several ways. Binding of calcium occurs by actine-myosine: re-uptake will take place into the sarcoplasmic reticulum, extrusion of calcium will occur across the sarcolemma and uptake of calcium will occur into the mitochondria. The latter process interferes with energy-rich phosphate production. From experiments by Lehninger et al[39,51], it became clear that the uptake of calcium into the isolated mitochondria took place at the expense of the phosphorylation of ADP, untill the concentration of calcium in the incubation medium had dropped back to the μM range. Later on, extensive research has been performed on the uptake and release of calcium from mitochondria.

Figure 2. Scheme of mitochondrial functioning, illustrating the production of ATP, and other processes powered by the proton gradient.



THE MEMBRANE OF THE MITOCHONDRION has embedded in it the enzymes and other components of the respiratory chain. The arrangement of the molecules, however, is not yet certain, and the model presented here is somewhat conjectural. Respiration begins with NADH, which gives up two electrons and a proton to flavin mononucleotide (FMN); another proton is picked up from the internal medium, so that the reduced form of the molecule ($FMNH_2$) carries two complete hydrogen atoms. The protons are expelled and the electrons return through an iron-sulfur protein (FeS) to the inner surface of the membrane. There the two electrons are donated to two molecules of ubiquinone (Q), each of which acquires a proton to form the semiquinone ($QH^\cdot$). Unlike the other components of the respiratory chain the quinones probably migrate as molecules through the membrane (*broken line*). The semiquinone takes on two more electrons from cytochrome *b* and with two more protons from inside the mitochondrion is converted into the fully reduced hydroquinone (QH_2). Each hydroquinone gives up one electron to cytochrome c_1 and releases the corresponding proton outside. The remaining two electrons are then returned to the cycle through cytochrome *b* and the last two of six protons are released. Finally the two electrons deposited with cytochrome c_1 pass through cytochromes *c*, *a* and a_3 to oxygen, which is thereby reduced to water. The proton circuit is completed by the F_1-F_0 complex, where each two protons driven inward bring about the synthesis of one ATP molecule. Other processes are also powered by the proton gradient. They include the reduction of $NADP^+$ by NADH, the transport of calcium (Ca^{++}) and sodium (Na^+) ions and the exchange of ADP for ATP. The illustrations on this page and the next two pages are based on drawings made by Maija V. Hinkle.

From: Hinkle P.C., McCarty R.E. How cells make ATP. Scientific American, march 1978; 238:109 (with permission).

The mitochondria appear to possess a separate uptake and release mechanism, each acting in one direction only[48]. The uptake of calcium is described as a chemiosmotic process, i.e. chemical reactions creating a concentration gradient over a membrane. This gradient creates an electrical potential over the mitochondrial inner membrane. This potential is able to drive the uptake of calcium into the mitochondrion, even in the respiratory blocked mitochondrion. Uptake of calcium is coupled to the extrusion of H^+ ions out of the matrix of the mitochondrion. This process is in electrical equilibrium (see figure 2).

However, continuous energy production is necessary to preserve the electro-chemical gradient over the mitochondrial inner membrane. The energy is derived from the electron transport chain. Calcium accumulation is indirectly coupled to electron transportation in the respiratory chain. When the intramyoplasmic calcium is increased beyond the physiological range, the energy of the electron transport chain will be used for the accumulation of calcium in the matrix of the mitochondrion and the coupled extrusion of H^+ ions into the sarcoplasma. The total capacity to accumulate calcium in mitochondria exceeds all other cellular components capacity[52]. It has been shown by Carafoli et al[52] in heart muscle that at physiological conditions most of the increased

intracellular amount of calcium is handled by the SR. However, during calcium overloading, mitochondria contribute substantially to the removal of the calcium. The mitochondrial uptake even exceeds the amount of calcium accumulated in the SR. It has been calculated that at an intracellular concentration of Ca^{2+} of 10^{-5} M, 50% of this amount is taken up by the mitochondria and about 46% by the SR; the sarcolemma extrudes only 4%. Under more physiological conditions (10^{-6} M Ca^{2+}) in the presence of Mg^{2+}, only 6% is accumulated by the mitochondria and 88% by the SR, whereas the sarcolemma extrudes about 5%. The maximal influx capacity of calcium into the mitochondria is about 10^{-7} M Ca^{2+}/mg protein/min at 25 ^{o}C in heart mitochondria. In the presence of Pi, it is increased to 6.10^{-7} M Ca^{2+}/mg protein/min at 25 ^{o}C . Therefore, the presence of Pi in the cytosol (originating from the hydrolysis of ATP) stimulates the calcium uptake.

Calcium uptake velocity into mitochondria obtained from MH susceptible pigs, was significantly lower compared to mitochondria from control pigs[45]. At a concentration of 2.10^{-4}M Ca^{2+} (far in the saturating range) the uptake into mitochondria from the MH susceptible pigs occurred at a significantly lower velocity (10^{-7}M Ca^{2+}/mg protein/min vs. $4x10^{-7}$M).

Also the mitochondrial binding capacity for Ca^{2+} MH

susceptible pigs was significantly lower than that in controls: $3.6.10^{-7}$ M Ca^{2+}/mg protein, and $5.6.10^{-7}$ M Ca^{2+}/mg protein respectively [44]. The additon of halothane produced a slight reduction in the uptake velocity of calcium into mitochondria (obtained from both types of pigs). All these experiments have been performed at 37 °C. Another study on calcium accumulation in porcine mitochondria at 25 °C [44], showed the same difference in calcium uptake capacity as stated above. This difference in total uptake capacity may be explained by a different pre-existent loading of the mitochondria with calcium. It has been shown by Cheah and Cheah [53], that mitochondria, obtained post mortem, have a higher endogenous concentration of calcium in the MH susceptible pigs as compared to the control ones. (In the MH susceptible pig mitochondria: 83.10^{-9}M/mg protein, in the control pigs mitochondria: 45.10^{-9}M/mg protein). However, the difference in the pre-existent loading may be explained by the method of studying: all muscle samples were taken post mortem, within 15 minutes after stunning. During this period calcium may already have entered the mitochondria, due to the stress inducing period just before slaughter. The differences found in the uptake velocity and uptake capacity of calcium in the mitochondria, are difficult to relate to the genesis of MH. Only the uptake of calcium into the mitochondria has been studied, however at unphysiologically high concentration. Because it is

not known which actual calcium concentration is reached during an MH crisis in the sarcoplasma, it is difficult to relate the findings of the calcium kinetic study to the in vivo situation. Only a continuously increased concentration of calcium, exceeding the physiological concentrations of calcium during muscular contraction is able to produce the concentrations, mentioned in the studies on calcium kinetics. The origin of this very high amount of calcium can be either the SR or an influx over the sarcolemma into the sarcoplasma.

Literature :

1. Gronert G. Malignant Hyperthermia. Anesthesiology 1980: 53, 395-423.
2. Artru A A, Gronert G. Cerebral metabolism during porcine malignant hyperthermia. Anesthesiology 1980: 53, 121-126.
3. Harrison G G. Porcine malignant hyperthermia. In: Malignant hyperthermia, Britt B A ed. Int Anesthes Clin 1979, vol 17 no. 4, 25-61.
4. Lucke J N, Hall G M, Lister D. Porcine malignant hyperthermia, I: metabolic and physiological changes. Br J Anaesth 1976: 48, 297-304.
5. Hall G M, Lucke J N, Lister D. Malignant hyperthermia pearls out of swine? Br J Anaesth 1980: 52, 165-171.
6. Lucke J N, Denny H, Hall G M, Lovell R, Lister D. Porcine malignant hyperthermia. VI: the effects of bilateral adrenalectomy and pretreatment with bretylium on the halothane induced response. Br J Anaesth 1978: 50, 241-245.
7. Gronert G A, Milde J H, Theye R A. Role of sympathetic activity in porcine malignant hyperthermia. Anesthesiology 1977: 47, 411-415.
8. Gronert G A, Milde J H, Taylor S R. Porcine muscle responses to carbachol, alpha- and beta adrenoceptor agonists, halothane or hyperthermia. J Physiol 1980: 397, 319-333.
9. Cohen P. The role of protein phosphorylation in neural

and hormonal control of cellular activity. Nature 1982: 296, 613-620.

10. Cheung W Y. Calmodulin plays a pivotal role in cellular regulation. Science 1980: 207, 19-27.
11. Means A R, Dedman J R. Calmodulin an intracellular calcium receptor. Nature 1980: 285, 73-77.
12. Editorial. Calmodulin-ubiquitous cell regulator. Lancet 1982: ii, 192-193.
13. Walsh M P. Calmodulin and its roles in skeletal muscle function. Can Anaesth Soc J 1983: 30, 390-398.
14. Lorkin P A, Lehmann H. Malignant hyperthermia in pigs: a search for abnormalities in Ca^{2+} binding proteins. FEBS lett 1983: 153, 81-87.
15. Gallant E M, Godt R E and Gronert G A. Role of plasma membrane of skeletal muscle in malignant hyperthermia. Muscle and Nerve 1979: 24, 491-494.
16. Gallant E M, Gronert G A and Taylor S R. Cellular membrane potentials and contractile treshold in mamalian skeletal muscle susceptible to malignant hyperthermia. Neurosci Lett 1982: 28, 181-186.
17. Bryant S H and Anderson I L. Mechanical inactivation and electrophysiological properties of intercostal muscle fibres from malignant hyperthermia susceptible (MHS) pigs. Neurosci Abstr: 1977: 3, 213.
18. Floyd K. The physiology of striated muscle. Br J Anaesth 1980: 52, 111-121.
19. Guyton A C. Textbook of medical physiology. 6th. ed. Philadelphia, London, Toronto: W B Saunders 1981:

122-137.

20. Endo M. Calcium release from the sarcoplasmic reticulum. Physiol Rev 1977: 57, 71-108.
21. Howell J N. A lesion of the transverse tubules of skeletal muscle. J Physiol 1969: 201, 515-553.
22. Kaminer B. Effect of heavy water on different types of muscle and glycerol extracted psoas fibres. Nature 1960: 185, 172-173.
23. Okumura F, Crocker B D,Denborough M A. Site of the muscle cell abnormality in swine susceptible to malignant hyperpyrexia. Br J Anaesth 1980: 52, 377-383.
24. Rosenberg H. Sites and mechanisms of action of halothane on skeletal muscle function in vitro. Anesthesiology 1979: 50, 331-335.
25. Morgan K G, Bryant S H. The mechanism of action of dantrolene sodium. J Pharmacol Exp Ther 1977: 201-138-147.
26. De Meis L, Inesi G. The transport of calcium by sarcoplasmic reticulum and various microsomal preparations.
In: Carafoli E, ed. Membrane transport of calcium. New York, Academic Press. 1982: 141-186.
27. Batra S. The effects of drugs on calcium uptake and calcium release by mitochondria and sarcoplasmic reticulum of frog skeletal muscle. Biochem Pharmacol 1974: 23, 89-101.

28. Martonosi A, Feretos R. Sarcoplasmic reticulum I. The uptake of Ca^{2+} by sarcoplasmic reticulum fragments. J Biol Chem 1964: 239, 648-658.

29. Gronert G, Heffron J J A, Taylor S R. Skeletal muscle sarcoplasmic reticulum in porcine malignant hyperthermia. Europ J Pharmacol 1979: 58, 179-187.

30. Berman M C, McIntosh D B, Kench J E. Proton inactivation of Ca^{2+} transport by sarcoplasmic reticulum. J Biol Chem 1977: 252, 994-1001.

31. Nelson T E, Bee D E. Temperature perturbation studies of sarcoplasmic reticulum from malignant hyperthermia pig muscle. J Clin Invest 1979: 64, 895-901.

32. Verburg M P, Oerlemans F T J J, van Bennekom C A, Gielen M J M, de Bruyn C H M M, Crul J F. In vivo induced malignant hyperthermia in pig. I: Physiological and biochemical changes and the influence of dantrolene sodium (Acta Anaesth Scand, in press).

33. Kalow W, Britt B W, Terreau M E. Metabolic error of muscle metabolism after recovery from malignant hyperthermia. Lancet 1970: ii 895-989.

34. Endo M. Calcium release from the sarcoplasmic reticulum Physiol Rev 1977: 57, 71-108.

35. Wood D S. Human skeletal muscle: analysis of Ca^{2+} regulation in skinned fibres using caffeine. Exp Neurol 1978: 58, 218-230.

36. Endo M, Yagi S, Ishizuka T, Horiuti K, Koga Y, Amaha K. Changes in the Ca^{2+} induced Ca^{2+} release mechanism in the sarcoplasmic reticulum of the muscle from a patient

with malignant hyperthermia. Biom Res 1983: 4, 83-92.

37. Strobel G E, Bianchi C P. An in vitro model of anesthetic hypertonic hyperpyrexia, halothane, caffeine, induced muscle contractures: prevention of contracture by procainamide. Anesthesiology 1971: 35, 465-473.

38. Cohen P J. Effect of anesthetics on mitochondrial function. Anesthesiology 1973: 39, 153-164.

39. Lehninger A L. Biochemistry, 2nd edition. New York: worth 1975: 509-542.

40. Miller R N, Hunter F E. The effect of halothane on electron transport, oxydative phsophorylation and swelling in rat liver mitochondria. Molec Pharmacol 1970: 6, 67-77.

41. Wilson R D, Nichols R J, Dent T E, Allen C R. Disturbance of the oxydative phosphorylation mechanism as a possible etiological factor in sudden unexplained hyperthermia occuring during anesthesia. Anesthesiology 1966: 27, 231.

42. Brooks G A, Cassens R G. Respiratory functions of mitochondria isolated from stress susceptible and stress resistant pigs. J Anim Sci 1973: 37, 688-691.

43. Eikelenboom G, Van den Berg S G. Mitochondrial metabolism in stress susceptible pigs. J Anim Sci 1973: 37, 693-696.

44. Britt B A, Endrenyi L, Cadman D L, Ho Man Fan, Fung H Y K. Porcine malignant hyperthermia: effects

of halothane on mitochondrial respiration and calcium accumulation. Anesthesiology 1975: 42, 292-300.

45. Gronert G A, Heffron J J A. Skeletal muscle mitochondria in porcine malignant hyperthermia: respiratory activity, calcium functions, and depression by halothane. Anesth Analg 1979: 58, 76-81.
46. Britt B A, Kalow W, Gordon A, Humphrey J G, Rewcastle N B. Malignant hyperthermia: an investigation of five patients. Can Anaesth Soc J 1973: 20, 431-467.
47. Mitchelson K R, Hird F J R. Effect of pH and halothane on muscle and liver mitochondria. Am J Physiol 1973: 225, 1393-1398.
48. Nicholls D, Åkerman K E O. Mitochondrial calcium transport Biochim Biophys Acta 1982: 683, 57-88.
49. Åkerman K E O, Nicholls D G. Physiological and bioenergetic aspects of mitochondrial calcium transport. Rev Physiol Pharmacol 1983: 95, 149-201.
50. Cheah K S, Cheah A M. Mitochondrial calcium transport and calcium activated phospholipase in porcine malignant hyperthermia. Biochim Biophys Acta 1981: 634, 70-84.
51. Lehninger A L. Mitochondria and calcium ion transport. Biochem J 1970: 119, 129-138.
52. Carafoli E. The transport of calcium across the inner membrane of mitochondria. In: Carafoli E, ed. Membrane transport of calcium. London: Academic Press, 1982: 109-139.
53. Cheah K S, Cheah A M. Mitochondrial calcium transport and calcium activated phospholipase in porcine malignant hyperthermia. Biochim Biophys Acta 1981: 634, 70-84.

INDEX CHAPTER 4 :

Screening methods to detect susceptibility to malignant hyperthermia in humans

CHAPTER 4 : SCREENING METHODS TO DETECT SUSCEPTIBILITY TO MALIGNANT HYPERTHERMIA IN HUMANS

4.1 Medical history

Several screening methods exist to evaluate MH susceptibility[1,2,3]. However, all have their restrictions and drawbacks.
Recording a family history is the first step in a screening procedure to evaluate MH susceptibility. It is of great importance to obtain the anaesthetic and surgical details of the MH episode a proband suffered from. The pre-operatieve temperature recording may give some clue to a febrile period in the peri-operative period. Also pre-operative laboratory data may be useful, especially the CK (creatine phosphokinase) value. The anaesthetic record should be screened regarding the administration of succinylcholine and one should look for the possible adverse reaction to succinylcholine, especially rigidity of the jaws or limbs. The administration of volatile anaesthetics has to be checked. One should also ask for the occurrence of muscular rigidity during or after the surgical intervention. The additional administration of muscular relaxants, exceeding the normal "topping up dose", should raise suspicion. If possible, one should interview the anaesthesist concerned about the details during the surgical procedure.
An arterial bloodgas analysis taken during the surgical intervention is very helpful in establishing the diagnosis of MH, especially high P_aCO_2 and increased base deficit.

The post-operative period has to be checked for excessive

temperature elevations, acid-base disturbances as well as muscular rigidity or neurological signs like coma or stupor. One should inform about complaints of painful, swollen muscles during the post-operative period. It should be remembered that succinylcholine alone may produce painful muscles in healthy subjects too.
The presence of myoglobinuria in the post-operative period is a sign of rhabdomyolysis. However, a recent study has revealed a high incidence of myoglobinuria in healthy humans receiving succinylcholine during general anaesthesia[4]. In the immediate post-operative period one should check CK values as well as serum K^+ levels.
A muscle biopsy taken during an MH episode may provide useful data, especially on morphological examination. However, taking a muscle biopsy during an MH crisis is not very appropriate due to the life-threatening condition of the MH crisis and the low priority of the muscle biopsy after all.

4.2 Physical examination.

Physical examination of MH susceptible subjects have not revealed any specific physical abnormalities. Some subjects are rather muscular and some may complain about spontaneous muscular cramps[3]. In MH susceptible children a high incidence has been observed of squint[3]. Some show a kypho-scoliosis, others hypermobility of the finger joints, shoulder or patella[3]. An increased incidence is observed of abdominal hernias.

A syndrome has been described by King and Denborough (1970) of which MH can be a feature[5]. It is characterised by a slowly progressive myopathy, short stature, kyphoscoliosis, pectus carinatum, cryptorchism, malar hypoplasia and downward slant of the palpebral fissure[5]. Motoric development is delayed, but intellectual development is normal. In some cases increased CK levels are measured. All cases described having this syndrome were children below the age of 15.

Another disease associated with MH is the so called "central core" disease[6]. This disease is diagnosed by microscopical examiniation of a skeletal muscle biopsy. In these muscle specimens a central area in type I fibres is poorly stained in contrast to the surrounding muscle. The core has lost its normal architecture of skeletal muscle, and there is a disarrangement of myofilaments.
On physical examination of such patients, no abnormalities are found[6].

4.3 Serological examination: Creatine phosphokinase (CK)

Initially, creatine phosphokinase (CK) was believed to be a useful diagnostic parameter for MH susceptibility[7,8,9]. However, further examination of a number of patients after surviving an MH crisis did not show increased activity levels of the enzyme several months after the MH crisis (false negatives). In about 70% of MH susceptible subjects,

CK levels are increased when measured after restraining from physical exercise[2]. The evaluation of the CK is useful as a basic screening method. It has even been suggested that when CK level is elevated in a close relative of a proven MH case, MH susceptibility may be considered to be present[1].

In contrast, elevated CK concentration may also be present in the absence of MH susceptibility (false positives). These findings urged to look for a more specific test to detect MH susceptibility. The investigations by Kalow et al[10] have led to the discovery of an increased sensitivity of skeletal muscle of MH susceptible subjects to halothane and caffeine.

4.4.0 The in vitro caffeine contracture test (CCT)

The in vitro caffeine contracture test (CCT) is a pharmacological test, based on the increased sensitivity to caffeine and/or halothane[10,11] of skeletal muscle from MH susceptible subjects. A muscle biopsy is taken from the M.vastus medialis or M.vastus lateralis, using either general anaesthesia or a loco-regional anaesthetic technique.
A muscle biopsy is taken of about 3 x 0.5 x 0.5 cm.
This sample size makes this test unsuitable for children below the age of 10-12 years.

The muscle sample is transported to the laboratory in a Krebs-Ringer solution at room temperature, aerated with carbogen, pH 7.4. In the laboratory, intact muscle fibres are prepared from the muscle specimen and fixed in a tissue bath. One end is attached to a force displacement transducer, measuring iso-metric force at 37 ^{o}C. The tissue bath is continuously aerated with carbogen, pH 7.4., and the muscle fascicle is directly stimulated (2 msec, 0.1 Hz, supra-maximally) in order to evaluate viability. If after about 15 minutes stimulation no twitch response is measured, the muscle sample is considered not viable and discarded. After obtaining twitch responses the muscle sample is stretched to its optimal length-tension response.
Increasing concentrations of caffeine in Krebs-Ringer solution are added at intervals of 5 minutes.
Muscle samples taken from MH susceptible individuals respond at lower concentrations of caffeine with a contracture than controls.
The increase of base-line tension without relaxation is called a contracture, without a spontaneous tendency to relax. During the contracture no electrical activity is measured.

The original study by Kalow et al [10] on the discriminating concentration of caffeine and/or halothane, to establish MH susceptibility, has caused vivid debates on the interpretation of the CCT in relation to the genetics of MH.

This has already been discussed in chapter 2.3. on the genetics of MH.

4.4.1 Effect of temperature on the CCT.

In the initial description of Kalow et al[10], the in vitro caffeine contracture test was performed at 25 °C, according to historical physiological studies on frog skeletal muscle. Exposure to halothane at 37 °C induced a contracture in skeletal muscle obtained from MH susceptible humans[11,12]. However, on exposure to halothane at 25 °C no contracture was observed, neither in skeletal muscle obtained from MH susceptible humans[10,13], nor in skeletal muscle obtained from MH susceptible pigs[14,15].

The caffeine induced contractures also showed a temperature dependency: at 20 °C and 25 °C significantly lower contracture heights were observed, compared to caffeine induced contractures at 37 °C[14,16]. However, at the low temperature range skeletal muscle obtained from MH susceptible pigs or MH susceptible humans still showed a greater contracture height compared to muscle samples taken from controls[14,16].

The effect of halothane plus caffeine is far less temperature dependent: a study on porcine skeletal muscle did not show a difference in contracture height comparing the contracture height attained at either 25 °C or at 37 °C[15].
Still a significantly higher contracture height was attained in MH skeletal muscle as compared to skeletal muscle obtained from controls. Comparable results have been obtained with human

skeletal muscle [16]. Based on these observations, it has been agreed upon to perform the in vitro CCT at 37 °C due to the valuable results of the halothane exposure at 37 °C.

4.4.2 Effect of calcium on the CCT.

It is assumed, that calcium has an important role in the aetiology of MH[17,18]. Studies have also been performed on the influence of calcium on the in vitro muscle contracture test. In the absence of calcium at 37 °C, no contracture developed after exposure to halothane[15,19,20,21]. In the absence of calcium the halothane plus caffeine induced contracture was markedly reduced: 91% reduction in MH susceptible muscle and 78% in the control muscle[19]. Generally, contracture responses to caffeine decreased with lowering of calcium concentration in the muscle bath, concomitantly with the twitch depression[19,20,21], but still caffeine produced a contracture, even at zero concentration of calcium.

This twitch depression was also most markedly present in the MH susceptible muscle specimens[19]. The depression of the caffeine induced contracture on lowering of the calcium concentration of the muscle bath, was most pronounced in the low concentration range of caffeine (0.5-4.0 mM). Contractures induced by high concentrations of caffeine were less affected by a drop in the calcium concentration of the muscle bath [19]. In the low range of caffeine concentrations (0.5-4.0 mM) the critical calcium concentration was 0.5 mM in the MH susceptible muscle specimens: i.e. increasing the calcium concentration in the tissue bath to

higher concentrations, did not increase the contracture response. In the control specimens the critical calcium concentration was 1 mM [19]. The contractures induced at relatively high concentrations of caffeine (8-32 mM) were more calcium dependent in MH susceptible muscle specimens as compared to control muscle samples [19].

Therefore, the use of a calcium concentration of 2.5 mM in the in vitro CCT is well beyond the calcium concentrations limiting the response to the caffeine and/or halothane contracture test.

4.4.3 Effect of fascicle size on the CCT.

No constant fascicle size can be obtained for the investigation of the muscle samples. This is due to the need of intact viable muscle fascicles for the in vitro CCT. Muscle samples obtained will range in weight from 50 to 150 mg. This makes it necessary to investigate the influence of sample size on the outcome of the CCT. Muscle fascicles weighing 100-500 mg, no difference was measured in the caffeine specific concentration: i.e. the concentration of caffeine producing a one gram contracture of the muscle specimen[22].

However, in spite of the difference in sample size, the developed contracture appeared to be influenced most by the caffeine and/or halothane concentrations added. Furthermore, a sample weighing about 300 mg is able to produce a maximal contraction force in vitro of about 12 grams[23]. This means that an increase of the contraction force of 1 gram is well below the maximal possible contraction force of the muscle

specimen. Therefore, the observations on the development of contractures on exposure to caffeine are carried out at the low range of the dose response curve where the weight of the muscle specimen is not very critical.

4.4.4 The effects of different inhalation anaesthetics on CCT.

The use of different inhalation anaesthetics in the in vitro caffeine contracture test has revealed the potential triggering mechanism of all inhalational anaesthetics to induce MH[24,25]. The anaesthetics tested were: halothane, enflurane, isoflurane and methoxyflurane, all administered in vitro at one MAC (minimum anaesthetic concentration)[24]. All inhalation anaesthetics tested potentiated the caffeine contracture test in vitro. However, a graded difference was present in their potency to increase the caffeine contracture test in vitro. At one MAC, the results of potentiation are in decreasing potency: chloroform > halothane > cyclopropane > isoflurane > enflurane > diethylether. These observations are in accordance with the clinical observations that all volatile anaesthetics are potentially triggering agents of an MH crisis, all with a graded triggering potency.

Furthermore, studies on the effect of inhalation anaesthetics on directly and indirectly stimulated muscle have shown an augmentation of the twitches on exposure to one MAC of all anaesthetics studied[26]. A comparable potentiation capacity was observed of the anaesthetics studied: in decreasing potency: halothane > methoxyflurane > fluroxene > diethylether[26].

4.4.5 Dynamic performance of the in vitro test.

A useful extension of the CCT is to study the dynamic properties of the muscle specimen tested. This test is based on the observation of a decreased compliance (increased-rigidity) of MH susceptible muscle on exposure to halothane or caffeine[27]. Due to this decreased compliance, a greater tension will be achieved by stretching the muscle specimen over the same distance, as in the 0% halothane exposure[27].

The test run for each concentration of halothane consists of an equilibration period of 5 minutes to the halothane concentration concerned. The muscle sample is stretched over the same distance as at the zero % halothane procedure. After leaving the muscle sample for about 5 minutes, the sample is relaxed by returning to the original length.

Stepwise increasing concentrations of halothane are used: 0.5%, 1%, 2% and 3% v/v. In the control specimens no change is observed of either the slope or the plateau of muscle tension reached. In skeletal muscle of MH susceptible subjects a steeper slope and a higher plateau level of tension is reached, on exposure to halothane.

This test has recently been extended by performing the dynamic test using caffeine. In this way a further refinement will be obtained (Lund protocol) (see appendix).

4.4.6 The effect of composition of fibre types on the CCT.

Studies of the in vitro exposure of muscle to caffeine has

shown a fibre type dependent reaction pattern [28,29]. Skeletal muscle composed primarily of fibre type 1 muscle, has a lower contracture threshold to caffeine compared to skeletal muscle composed primarily of fibre type II [28,29]. These observations point to the necessity to include in the in vitro CCT a histological examination of the skeletal muscle specimen.

4.5 Adenosine triphosphate (ATP)-depletion test in muscle specimens.

The ATP-depletion test is an in vitro assay, based on the depletion of ATP in a muscle specimen exposed to halothane compared to a muscle specimen incubated at the same conditions without exposure to halothane [30,31,32]. The ATP-depletion is expressed as a ratio: ATP concentrations in muscle exposed to halothane 4% v/v divided by ATP concentrations in muscle not exposed to halothane. The incubation is performed at 37 ^{o}C for 30 minutes and aerated with carbogen. The ratio for skeletal muscle of controls is: 1.04 $\pm$ 0.05, indicating that in normal (control) subjects, there is no decrease of the ratio. In contrast, in MH subjects a significant decrease is measured in the ratio. These observations illustrate the effect of halothane on muscle metabolism in MH susceptible humans and MH susceptible pigs.

However, when studying the ATP-depletion in the porcine MH model, a considerable overlap became apparent between the ratios obtained from MH susceptible pigs and control pigs [32]. It was concluded that: "values of ATP-depletion probably fluctuate more due to experimental than due to biological

variability" [32].

The ATP-depletion test is not useful as a screening method, to detect MH susceptibility.

4.6 Additional in vitro muscle tests.

Additional investigations have been performed with isolated skeletal muscle preparations, exposed to a variety of chemicals. Several authors have used a high concentration of K^+ in the bathing medium in order to induce depolarization of the muscle cell membrane[33,34,35]. This depolarization bypasses the neuro-muscular junction. At concentrations higher than 20 mM K^+, a depolarization is induced, resulting in skeletal muscle contraction. In MH susceptible subjects, an bigger contracture force is measured compared to control muscle samples on exposure to K^+ [33,36,37,38].

A further screening procedure has been developed, using succinylcholine as a contracture inducing agent[33,36,37,38]. It is a rather equivocal method, as (almost) no intact neuro-muscular junctions will be present in the muscle sample, due to the biopsy technique, separating the muscle sample from its innervating neuron. It has been shown that a muscle sample taken from a control subject does not produce a contracture on addition of succinylcholine[39]. Mostly, succinylcholine is used in combination with caffeine to produce a contracture[39]. The use of succinylcholine in the screening of MH has not universally been accepted by centers performing MH screening tests [1,3].

4.7 Histological screening for MH.

No specific histological abnormality has been found in skeletal muscle, which might be used as diagnostic tool for MH[1,2,3,6]. However, a wide variety of histological abnormalities has been presented in human MH susceptible subjects.

The most common histological finding is the occurrence of internal nuclei[40]: over 3% of the nuclei are situated in the myoplasma. The internal nuclei have to be present in all muscle fascicles. On longitudinal examination of the internal nuclei, it was shown that they are present in short chains, different from the long and compacted chains seen in myotonia. No preference of fibre type was seen in the distribution of internal nuclei[40].

A second common finding is the presence of central cores[6,40,41]: loss of normal architecture in the core of the skeletal muscle fibres, leading to streaming of the myofilaments and desintegration. No preponderance of fibre type I is seen in MH cases with central cores, in contrast to those affected by the central core disease, having cores present only in fibre type I[6]. The amount of central cores has to exceed 3-4/ field of moderate magnification, to be of clinical significance, since in control humans about 2-3 central cores may be present[7,40].

A rather uncommon finding is the occurrence of very small fibres, sometimes angulated[42,43], or type IIB atrophy[44]. This may indicate a neurogenic disease; however, this is

still a matter of debate[45,46,47]. With respect to the innervation of skeletal muscle of MH susceptible humans, no abnormalities have been found, neither in the type of innervation nor in the neuro-muscular end-plates. MH as a manifestation of a denervating disease has been ruled out by these results.

4.8 Electromyographic examination.

The electromyographic examination of MH susceptible humans did not show consistent abnormalities[3]. A higher incidence of polyphasic action potentials has been reported[48], a sign for a myopathy. However, the incidence of abnormal electromyographic recordings is too low and shows too wide a variety of small abnormalities, being insufficient for a screening method.

4.9 Serum cholinesterase evaluations.

Extensive investigations have been performed on the possible relation between abnormal cholinesterase activity and MH[49]. It has been suggested that in patiens with a history of MH or a family history of MH, an increased incidence occurs of a "fluoride-resistant gene"[50]. It was claimed by these authors that in families with a history of MH, the frequency of such a "fluoride-resistant gene" will be about 100 times higher than in the control population. However, two other groups have reported data contradictory to the suggested increased incidence of the "fluoride-resistant gene"[51,52].

A major criticism on former investigation concerns the diagnostic criteria for establishing MH susceptibility: only a family history of MH or some kind of jaw stiffness during a surgical procedure was already considered as diagnostic for MH [50]. No in vitro skeletal muscle assay was performed to confirm the diagnosis of MH , questioning MH susceptibility.
Summarizing, the evaluation of serum cholinesterase activity can not be considered to be a useful diagnostic criterium to evaluate MH susceptibility, nor can it be used as a screening procedure.

4.10 Platelet bio-assay.

It has been reported by Solomons et al that human platelets can be used as a screening method [53,54,55]. It was claimed that exposure of platelets to halothane in vitro will cause a depletion of ATP in platelets from MH susceptible subjects. This depletion is far greater in the MH susceptible individuals than in controls. However, further investigation by Giger et al [56] showed that the ATP-depletion occurred to the same amount in the MH susceptible subjects as well as in controls. So, it was concluded that the platelet bio-assay does not seem a useful screening method to detect MH susceptibility.

4.11 Skeletal muscle enzymes.

4.11.1 Adenylate kinase: (E.C. 2.7.4.3)

The usefulness as a screening test for MH of enzymes regulating

energy metabolism has been suggested [57]. Initial studies of the enzyme adenylate-kinase seemed promising. This enzyme catalyzes the reversible formation of 1 mol ATP out of 2 mol ADP. The reaction scheme is: 2 ADP $\longleftrightarrow$ 1 ATP + 1 AMP. A deficiency of this enzyme might account for the depletion of ATP in skeletal muscle.
The first report on a deficiency of adenylate kinase in skeletal muscle in MH susceptible subjects had to be rejected on further investigations [58,59]. No basis was found for a deficiency of adenylate kinase, neither in MH susceptible humans [58] nor in MH susceptible pigs [59].

4.11.2 (Myo)Phosphorylase: (E.C. 2.4.1.1)

Studies on the enzyme phosphorylase-a as an indicator of MH susceptibility have also been promising [60]. This enzyme is the activated phosphorylase-b, catalysing glycogen breakdown. This activation of glycogen breakdown by phosphorylase-a is initiated by adrenergic stimuli, producing an increased concentration of the second messenger cyclic-AMP, activating the cascade of phosphorylase activity. The ratio phosphorylase-a/total phosphorylase was in the control population 2.6 $\pm$ 2.0. In the MH susceptible group 45.6 $\pm$ 26.2. However, in one case studied a false negative reaction was measured of the phosphorylase ratio as compared to the in vitro caffeine contracture test. A contra-expertizing investigation has led to an opposite conclusion [61]. MH susceptibility was confirmed by a medical history of a possible MH reaction and completed by an in-vitro test of skeletal muscle. In the control population the ratio phosphorylase-a/total phosphorylase showed a mean

value of 12.4 ± 1.9. The MH susceptible group a mean value of 14.5 ± 2.0. No statistically significant difference was measured between the two groups. It is concluded from this study that the phosphorylase ration can not be used as a screening method to detect MH susceptibility [61].

4.11.3 AMP-deaminase : (E.C. 3.5.4.6) (adenylate deaminase) This enzyme catalyzes the breakdown of AMP into IMP and NH_3. By decreasing the concentration of AMP, the energy charge can be kept at level. A deficiency of this enzyme will lead to an accumulation of AMP, and will inhibit the activity of the enzyme adenylate kinase by accumulation of the substrate AMP. In this way a reduction takes place in the ATP concentration. In a preliminary report, Fishbein et al [62] reported a normal level of the enzyme AMP-deaminase in an MH susceptible human.

From this observation it may be concluded that AMP-deaminase is not deficient in MH susceptible humans and can not be used as a screening method for MH.

4.12 Conclusion :

Screening for MH has to start with an extensive investigation of all anaesthesia related facts of a supposed MH crisis. One has to exclude a pre-existent muscular disease, producing skeletal muscle rigidity after administration of succinylcholine. Physical examination will not provide specific abnormalities in MH susceptible subjects. Elevation of serum CK values may be present in about 70% of all MH susceptible subjects. However, also false positive values may be seen.

At present, the best test to confirm MH susceptibility is the in-vitro test of a skeletal muscle sample exposed to increasing concentrations of caffeine or halothane. Skeletal muscle obtained from MH susceptible subjects shows an increased sensitivity to halothane and to caffeine, producing a contracture. In this way a differentation can be made between MH susceptible humans and those not susceptible to MH.

LITERATURE :

1. Gronert G A: Malignant hyperthermia. Anesthesiology 1980: 53, 395-423.
2. Britt B A, Kwong FH F, Endrenyi L: The clinical and laboratory features of malignant hyperthermia management - a review. In: Malignant hyperthermia, current concepts. Edited by EO Henschel. New York, Appleton-Century-Crofts, 1977: 9-47.
3. Britt B A: Pre-anesthetic diagnosis of malignant hyperthermia. In: Malignant hyperthermia. Edited by B A Britt. Int Anesthes Clin 1979: 17, 63-96.
4. McKishnie J D, Muir J M, Girvan D P. Anaesthesia induced rhabdomyolysis. Can Anaesth Soc J 1983: 30, 295-298.
5. Denborough M A, King J O, Ebeling P et al: Myopathy and malignant hyperpyrexia. Lancet ii, 1970: 1138.
6. Eng G D, Epstein S, Engel W K, McKay D W, McKay R. Malignant hyperthermia and central core disease in a child with congenital dislocating hips. Arch Neurol 1978: 35, 189-197.
7. Ellis F R, Clarke I M C, Modgill M, Currie S and Harriman D G F: Evaluation of creatine phosphokinase in screening patients for malignant hyperpyrexia. Br Med J 1975: 3, 511-513.
8. Isaacs H and Barlow M B: The genetic background to malignant hyperpyrexia revealed by serum creatine phosphokinase estimations in asymptomatic relatives. Br J Anaesth 1970: 42, 1077-1084.

9. Britt B A, Endrenyi L, Peters P L et al: Screening of malignant hyperthermia susceptible families by creatine phosphokinase measurements and other clinical investigations. Can Anaesth Soc J 1976: 23, 263-184.
10. Kalow W, Britt B A, Terreau M E and Haist C: Metabolic error of muscle metabolism after recovery from malignant hyperthermia. Lancet ii: 1970: 895-898.
11. Ellis F R, Keany N P, Harriman D G F, Sumner D W, Kyei-Mensah K, Tyrrel J H, Hargreaves J B, Parikh R K and Mulrooney P L: Screening for malignant hyperpyrexia. Br Med J 1972: 3, 559-561.
12. Moulds R F W, Denborough M A: Procaine in malignant hyperpyrexia. Br Med J 1972: 4, 526-528.
13. Nelson T E, Austin K L, Denborough M A: Screening for malignant hyperpyrexia. Br J Anaesth 1977: 49, 169-172.
14. Sullivan J S, Denborough M A: Temperature dependence of muscle function in malignant hyperpyrexia-susceptible swine. Br J Anaesth 1981: 53, 1217-1222.
15. Nelson T E, Bedell D M, Jones E W: Porcine malignant hyperthermia: effects of temperature and extra-cellular calcium concentration on halothane induced contracture of susceptible skeletal muscle. Anesthesiology 1975: 42, 301-306.
16. Britt B A, Endrenyi L, Scott E, Frodis W: Effect of temperature, time and fascicle size on the caffeine contracture test. Can Anaesth Soc J 1980: 27, 1-11.
17. Britt B A: Etiology and pathophysiology of malignant hyperthermia. Fed Proc 1979: 38, 44-48.

18. Denborough M A. Etiology and pathophysiology of malignant hyperthermia. In: Britt B A ed. Malignant Hyperthermia. Int Anesthes Clin 1979: 17, 11-23.
19. Nelson T E, Chausmer A B: Calcium content and contracture in isolated muscle of malignant hyperthermia in pigs. J Pharmacol Exp Ther 1981: 219, 107-111.
20. Moulds R F W, Denborough M A: Biochemical basis of malignant hyperpyrexia. Br Med J 1947: 2, 241-244.
21. Moulds R F W, Denborough M A: A study of the action of caffeine, halothane, potassium chloride and procaine on normal human skeletal muscle. Clin Exp Pharmacol Physiol 1974: 1, 197-209.
22. Britt B A, Endrenyi L, Scott E, Frodis W: Effect of temperature, time and fascicle size on the caffeine contracture test. Can Anaesth Soc J 1980: 27, 1-11.
23. Kalow W, Britt B A, Richter A: The caffeine test of isolated human muscle in relation to malignant hyperthermia. Can Anaesth Soc J 1977: 24, 678-694.
24. Britt B A, Endrenyi L, Frodis W, Scott E, Kalow W: Comparison of effects of several inhalation anaesthetics on caffeine induced contractures of normal and malignant hyperthermic skeletal muscle. Can Anaesth Soc J 1980: 27, 12-15.
25. Reed S B, Strobel G E: An in vitro model of malignant hyperthermia: differential effects of inhalation anesthetics on caffeine induced muscle contractures. Anesthesiology 1978: 48, 254-259.
26. Waud B E, Waud D R: Effects of volatile anesthetics on

directly and indirectly stimulated skeletal muscle. Anesthesiology 1979: 50, 103-110.

27. Ellis F R, Harriman D G F, Currie S, Cain P A: Screening for malignant hyperthermia in susceptible patients. In: Malignant hyperthermia, second international symposium, eds Aldrete J A, Britt B A. New York: Grune and Stratton, 1978: 273-285.
28. Nelson T E, Schochet S S. Malignant hyperthermia: a disease of specific myofibre type? Can Anaesth Soc J 1982: 29, 163-167.
29. Brownell A K W, Szabo M. The in-vitro caffeine contracture test: influence of the muscle histochemical profile on test results. Can Anaesth Soc J 1982: 29, 218-221.
30. Harrison G G, Saunders S J, Biebuyck J F, Hickman R, Dent D M, Weaver V, Terblanche J. Anaesthetic induced malignant hyperpyrexia and a method for its prediction. Br J Anaesth 1969: 41, 844-855.
31. Britt B A, Endrenyi L, Kalow W, Peters P L: The adenosine trophosphate (ATP) depletion test: comparison with the caffeine contracture test as a method of diagnosing malignant hyperthermia susceptibility. Canad Anaesth Soc J 1976: 23, 624-635.
32. Gronert G A: Muscle contractures and adenosine trophosphate depletion in porcine malignant hyperthermia. Anest Analg 1979: 58, 367-371.
33. Moulds R F W, Denborough M A: Biochemical basis of malignant hyperpyrexia. Br Med J 1974: 2, 241-244.
34. Moulds R F W, Denborough M A: Identification of suscep-

tibility to malignant hyperpyrexia. Br Med J 1974: 2, 245-247.

35. Moulds R F W, Denborough M A: A study of the action of caffeine, halothane, potassium chloride and procane on normal human skeletal muscle. Clin Exp Pharmacol Physiol 1974: 1, 197-209.

36. Gallant E M, Gronert G A, Taylor S R: Cellular membrane potentials and contractile threshold in mammaliam skeletal muscle susceptible to malignant hyperthermia. Neurosci Lett 1982: 28, 181-186.

37. Okumura F, Crocker B D, Denborough M A: Site of the muscle cell abnormality in swine susceptible to malignant hyperpyrexia. Br J Anaesth 1980, 52, 377-383.

38. Okumura F, Crocker B D, Denborough M A: Identification of susceptibility to malignant hyperpyrexia in swine. Br J Anaesth 1979: 51, 171-176.

39. Halsall P J, Ellis F R: A screening test for the malignant hyperpyrexia phenotype using suxamethonium induced contracture of muscle treated with caffeine, and its inhibition by dantrolene. Br J Anaesth 1979: 51, 753-755.

40. Harriman D G F. Preanesthetic investigation of malignant hyperthermia: microscopy. In: Malignant hyperthermia. Ed B A Britt. Int Anesthes Clin, 1979: 17, no 4, 97-117.

41. Frank J P, Harati Y, Butler IJ, Nelson T E, Scott C I: Central core disease and malignant hyperthermia syndrome. Ann Neurol 1980: 7, 11-17.

42. Isaacs H, Heffron J J A: Morphological and biochemical

defects in muscles of human carriers of the malignant hyperthermia syndrome. Br J Anaesth 1975: 47, 475-481.

43. Isaacs H, Barlow M B: Malignant hyperpyrexia: further muscle studies in asymptomatic carriers identified by creatine phosphokinase screening. J Neurol Neurosurg Psychiat. 1973: 36, 228-243.

44. Allen P D, Ryan J F, Sreter F A, Mabuchi K: Histochemical changes in malignant hyperthermia susceptible patients. Anesthesiology 1980: 53, S 252.

45. Harriman D G F, Ellis F R, Franks A J, Summer D W: Malignant hyperthermia myopathy in man: an investigation of 75 families. In: Second International Symposium on Malignant hyperthermia. Aldrete J A, Britt B A eds. New York: Grune & Stratton, 1978: 67-87.

46. Hull M T, Muller J, Albrecht W H: Morphologic abnormalities in a case of malignant hyperthermia. Anesthesiology 1978: 48, 223-228.

47. Brooke M H, Engel W K: The histologic diagnosis of neuromuscular diseases: a review of 79 biopsies. Arch Phys Med Rehabil 1966: 47, 99-121.

48. Steiss J E, Bowen J M, Williams C H: Electromyographic evaluation of malignant hyperthermia susceptible pigs. Am J Vet Res 1981: 42, 1171-1176.

49. Hall G M: Editorial: plasma cholinesterase and malignant hyperthermia. Br J Anaesth 1981: 53, 199-200.

50. Whittaker M, Britten J J: Malignant hyperthermia and the fluoride-resistant gene. Br J Anaesth 1981: 53, 241-244.

51. Evans R T, Iqbal J, Ellis F R , Halsall P J, Dietz A A,

Lubrano T, Rubinstein H M: Collaborative study of the frequency of the fluoride-resistant cholinesterase variant in patients with malignant hyperpyrexia. Br J Anaesth 1981: 53, 245-247.

52. Ording H, Hanel H H, Viby-Mogensen J: Plasma cholinesterase and malignant hyperthermia. Br J Anaesth 1981: 53, 317.

53. Solomons C C, Tan S, Aldrete J A: Platelet metabolism and malignant hyperthermia. In: Second International Symposium on malignant hyperthermia. Aldrete J A, Britt B A eds. New York: Grune & Stratton, 1978: 221-225.

54. Solomons C C, Masson N C: A platelet halothane bio-assay for malignant hyperthermia. Anesthesiology 1982: 57, A 225.

55. Mahowald H C, Masson M B, Solomons C C: Regulation of dantrolene pretreatment in malignant hyperthermia susceptible patients using a platelet bio-assay. Anesthesiology 1982: 57, A 226.

56. Giger U, Kaplan R F: Halothane induced ATP-depletion in platelets from patients susceptible to malignant hyperthermia and from controls. Anesthesiology 1983: 58, 347-352.

57. Schmitt J, Schmidt K, Ritter H. Heriditary malignant hyperpyrexia associated with muscle adenylate kinase deficience. Hum Genet 1974: 24, 253-257.

58. Cerri C G, Willner J H, Britt B A, Wood D S. Adenylate kinase deficiency and malignant hyperthermia. Hum Genet 1981: 57, 325-326.

59. Marjanen L A, Shaw D C, Denborough M A. Comparison of

adenylate kinase from normal and malignant hyperpyrexic porcine muscle. Biochem Med 1983: 29, 164-170.

60. Willner J H, Wood D S, Cerri C, Britt B A. Increased myophosphorylase-a in malignant hyperthermia. New Engl J Med 1980: 303, 138-140.

61. Traynor C A, Van Dyke R A, Gronert G A. Phosphorylase ratio and susceptibility to malignant hyperthermia. Anesth Analg 1983: 62, 324-326.

62. Fishbein W N, Armbrustmacher V W, Griffin J L. Skeletal muscle adenylate deaminase, adenylate kinase and creatine kinase in myo-adenylate deaminase deficiency and malignant hyperthermia. Clin Res 1980: 28, A 288.

CHAPTER 5

In vivo induced malignant hyperthermia in pigs. I: Physiological and biochemical changes and the influence of dantrolene sodium.

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In Vivo Induced Malignant Hyperthermia in Pigs. I. Physiological and Biochemical Changes and the Influence of Dantrolene Sodium

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The effects of an induced malignant hyperthermia (MH) crisis have been studied in the intact pig Both physiological and biochemical changes in skeletal muscle were studied MH was induced with 3% halothane plus a bolus injection of succinylcholine In the prechallenge period a significant difference was observed in the concentration of certain muscle metabolites, comparing the MH-susceptible (MH^+) with the non-susceptible (MH^-) pigs A lower level was measured for phosphocreatine (PCr), inosine monophosphate (IMP) and an increased level of lactate and creatine (Cr) in the susceptible pigs (MH^+) The challenge caused a significant reduction of the level of PCr and adenosine in MH^+ pigs, compared to the prechallenge period After administration of dantrolene sodium, a significant decrease was measured in the level of lactate, compared to the prechallenge period as well as during the challenge In contrast, in the control pigs no significant changes were observed in muscle metabolites, either after induction of MH or after the administration of dantrolene sodium Enzyme activity determinations of muscle adenylate kinase and adenosine monophosphate (AMP)-deaminase did not show any difference in activity either before or during the MH crisis or after treatment with dantrolene sodium The earliest physiological change during an induced MH crisis in our study was the rapid increase of the end-tidal CO_2 Within 5 min after MH induction, end-tidal CO_2 was doubled It is concluded that the monitoring of the end-tidal CO_2 is essential to diagnose MH at a very early stage

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Key words Adenosine triphosphate, adenylate kinase, AMP-deaminase, dantrolene sodium, halothane, malignant hyperthermia; muscle metabolites, phosphocreatine, pigs, skeletal muscle, succinylcholine

Malignant hyperthermia (MH) is a pharmacogenetic disorder with a high mortality of about 30%, in spite of the availability of a specific drug (dantrolene sodium) to treat this disease (1). The signs and symptoms of an MH crisis include skeletal muscle rigidity, rapid increase of body temperature, cardiac arrhythmias, cyanosis, severe metabolic acidosis, hyperkalaemia and elevation of the serum concentration of the muscle enzyme creatine phosphokinase (CK) (1). However, most of these symptoms occur at a relatively late phase of the MH crisis, and so once these signs appear clinically, irreversible damage may have occurred, especially brain damage due to the severe acidosis and hypoxia. Therefore, the need for a specific diagnostic parameter for MH in a very early phase of the MH crisis is obvious.

The aim of the present study was to evaluate biochemical changes as well as physiological changes during an induced MH crisis in the porcine MH model.

In this combined set-up we studied the MH crisis in the intact animal, in contrast to most biochemical studies on MH, done *in vitro* on isolated organelles. In this way we were able to relate biochemical changes in skeletal muscle with some physiological changes during the induction of the MH crisis and after treatment with dantrolene sodium. This further enabled us to evaluate also the effects of dantrolene sodium on skeletal muscle metabolism.

In order to study pathophysiological changes in MH, the pig model was used, which is an accepted model for the MH crisis in humans (2). The present study as a whole involves pathophysiological, biochemical and electronmicroscopic aspects of the MH crisis.

The first paper is mainly concerned with the changes in skeletal muscle, as it has been supposed that this organ is primarily involved in the MH crisis (1). Serological and pathophysiological changes are described, as well as

changes in the levels of high energy compounds in skeletal muscle Especially the changes in adenine nucleotides, phosphocreatine (PCr) and lactate have been evaluated, because of their crucial role in muscle physiology The second paper of the study comprises the evaluation of aerobic metabolism of the mitochondria before and during the MH crisis (3) This was done in order to obtain a better insight into mitochondrial functioning during the MH crisis, in relation to the heat production and changes in the high energy compounds in skeletal muscle The mitochondria were isolated before and during the MH crisis

The third paper (4) involves a study of the changes in calcium localization, using electronmicroscopic and electronmicroprobe techniques (5) Emphasis is put on the study of the calcium movements in the muscle cells during the MH crisis and after reversal of the MH crisis by dantrolene sodium The role of calcium in the aetiology of MH has been suggested, causing the increased metabolism and muscle rigidity (1)

MATERIAL AND METHODS

Ten Dutch Landrace pigs were used in this study At the age of about 6 weeks the pigs were tested with a halothane inhalation challenge in order to evaluate MH susceptibility (6) Five MH susceptible pigs (MH^+) and five control pigs (MH^-) were studied The MH susceptible group consisted of three littermates and two littermates from a different litter The control pigs originated from two different litters, i e three littermates and two littermates The procedures used in the experiment were in accordance with the legal regulations for the care and use of laboratory animals

After overnight fasting, premedication was given to the pig to be studied Fentanyl 0 1 mg and dehydrobenzperidol 5 mg was given i m (Janssen Pharmaceutica, Beerse, Belgium) A sleep dose of pentobarbital (25 mg/kg bw) was given i v , 15 min after premedication Without using a muscle relaxant, the pig was intubated and ventilated with a mixture of oxygen and air, F_{IO_2} 0 4

A constant volume ventilator (Engström) was used to set the minute volume to achieve an arterial CO_2 pressure of 5 0-6 0 kPa Expiratory CO_2 was continuously measured by means of a capnograph, type Mark II (Gould Godart, Bilthoven, The Netherlands) A carotid artery was cannulated in order to trace arterial blood pressure continuously and to draw blood samples to perform arterial blood gas analysis and serological analysis Core temperature was measured rectally and muscle temperature was measured in skeletal muscle of a hind limb Supplementary pentobarbital was given to keep the pig asleep and fentanyl was given in case of painful procedures

In the experimental procedure three states are to be discerned *State A* is the sampling time after completing all preparatory manipulations, described in the preceding text, and after obtaining a stable arterial blood pressure and a constant arterial CO_2 pressure *State B* is the sampling time after a 10 min challenge with halothane 3% plus a bolus injection of succinylcholine 2 mg/kg bw, given at the start of the challenge *State C* is the sampling time 30 min after administration of dantrolene sodium, 1 mg/kg bw, and hyperventilation with oxygen, F_{IO_2} 1 0

State A

At this time, several muscle biopsies were taken from the M biceps femoris One muscle sample was used to perform the *in vitro* halothane and caffeine contracture test (7-8) Another sample was immediately frozen in liquid nitrogen This muscle sample was used to evaluate levels of muscle metabolites by means of iso-tachophoresis (9) and to measure the activities of the muscle enzymes adenylate kinase (E C 2 7 4 3) (10) and AMP deaminase (E C 3 5 4 6) (10) Two further muscle samples were taken one to study mitochondrial functions and the second one to perform the electronmicroscopic studies

Blood gas analysis was performed with a Corning blood gas analyser (type pH Bloodgas 165, Medfield, Mass USA) Furthermore a blood sample was drawn to analyse serum lactate, inorganic phosphate (P_i), Na^+ K^+, Mg^{2+}, Ca^{2+} and the serum enzymes CK and lactate dehydrogenase (LDH), using standard laboratory techniques

The protein content of the muscle samples was measured, using the method described by Lowry et al (11)

Free creatine contents were measured in a neutralized perchloric acid extract of a muscle specimen, applying the enzymatical system described by Jaworek et al (12), with the addition of creatine kinase and adenosine triphosphate (ATP)

State B

At the start of the challenge (called t_0), a bolus of succinylcholine (2 mg/kg bw) was given together with halothane 3% v/v At the end of the 10 min challenge, state B was reached (t_{10}) Muscle biopsies were taken for analysis of muscle metabolites, mitochondrial functions and electronmicroscopy Furthermore blood samples were taken to examine blood gas values as well as the serological parameters, as mentioned in state A Halothane administration was terminated at t_{10} and F_{IO_2} increased to 1 0, without changing minute volume Arterial blood gas was measured 5 min later, in order to evaluate a possible spontaneous recovery from the MH crisis

State C

Dantrolene sodium was administered, 1 mg/kg bw at t_{15} Hyperventilation was also performed, F_{IO_2} 1 0

State C (t_{45}) is reached 30 min after administration of dantrolene and continued hyperventilation with oxygen, F_{IO_2} 1 0

Muscle biopsies were taken to analyse muscle metabolites, muscle enzymes as well as a biopsy for electron microscopic studies Arterial blood samples were taken to measure blood gas values as well as serum electrolytes and serum enzymes

After closing the incisions, the endotracheal tube was removed and the pig transported to the recovery room

Statistics

The statistical method used was Student's *t*-test for unpaired values when comparing the results between the MH^+ and MH^- groups The *t*-test for paired values was used for evaluation within each group

RESULTS

1 Physiological data

Exposure to the halothane + succinylcholine challenge confirmed in all pigs the halothane inhalation test, discriminating the MH-susceptible pigs from the MH^- pigs Dantrolene was effective in curing the MH crisis, as all MH-susceptible pigs survived the challenge

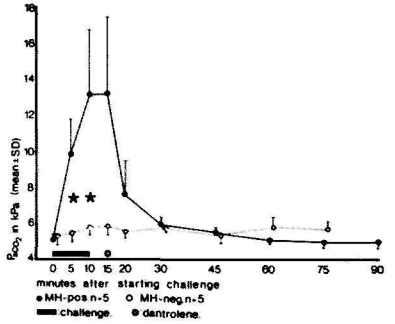

Fig. 1. Arterial CO_2 pressure plotted against the time period of the experiment. The challenge with a bolus injection of succinylcholine 2 mg/kg bw and exposure to halothane 3% v/v, is shown by the bar in the figure. Start of the challenge at t_0. From t_{10}–t_{15} ventilation with 100% O_2 with unchanged minute volume. At t_{15} dantrolene sodium is administered 1 mg/kg bw and hyperventilation with 100% O_2 is started. No other supportive measures are taken. Statistically significant differences between groups: ★ : $P<0.05$.

The effect of the challenge on Pa_{CO_2} is shown in Figure 1. Five minutes after starting the challenge a significant ($P=0.029$) increase of Pa_{CO_2} occurred in the MH^+ pigs. No change was noted in Pa_{CO_2} in the MH^+ pigs on termination of the challenge. The administration of dantrolene sodium, however, produced a decrease of Pa_{CO_2} within 5 min after infusion of dantrolene. About 15 min after dantrolene administration, Pa_{CO_2} returned to the pre-challenge levels. The MH^- pigs did not show a change in Pa_{CO_2} either during the challenge or after the administration of dantrolene sodium.

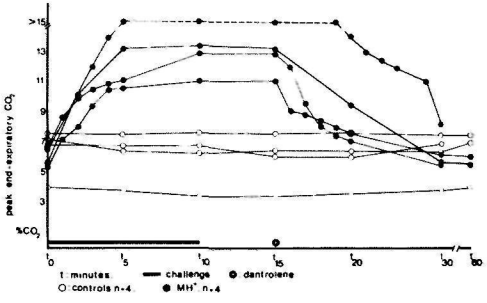

Fig. 2. Registration of the end-expiratory CO_2 concentration. Start of the challenge at t_0. The challenge consisted of a bolus injection of succinylcholine 2 mg/kg bw plus halothane 3% v/v for 10 min. Halothane administration stopped at t_{10}; ventilation with unchanged minute volume, Fi_{O_2}: 1.0. Administration of dantrolene sodium 1 mg/kg bw at t_{15}, and hyperventilation, Fi_{O_2}: 1.0.

The end-expiratory CO_2 in the MH-susceptible pigs showed a significant ($P=0.001$) increase during the challenge (Fig. 2). Within 5 min after starting the challenge the end-expiratory CO_2 was doubled. After termination of the challenge no decrease was measured. On administration of dantrolene sodium, a significant ($P=0.01$) decrease was seen within 5 min. The end-expiratory CO_2 in the MH^- pigs did not change, either during the challenge or after the administration of dantrolene sodium (Fig. 2).

A typical tracing of the end-expiratory CO_2 is shown in Figure 3. The administration of succinylcholine + halothane caused an immediate increase of CO_2 production. After 4 min a concentration of 10% was reached. The maximal level measured during this experiment reached far beyond the 10% level.

The challenge caused a significant ($P=0.03$) decrease of arterial pH within 5 min in the MH^+ pigs (Fig. 4). On administration of dantrolene sodium, a return to the pre-challenge level was reached after about 60 min. In the MH^- pigs no change was observed in arterial pH, either during the challenge or on the administration of dantrolene sodium (Fig. 4).

In the MH^+ pigs a significant ($P=0.005$) increase was seen in both muscle temperature and core temperature (Fig. 5). Core temperature lagged behind muscle temperature during the experiment. After the administration of dantrolene sodium, no further increase of temperature was measured, in muscle or in core temperature. In the MH^- pigs a slight decrease (0.2°C) was measured in core temperature for the first 30 min.

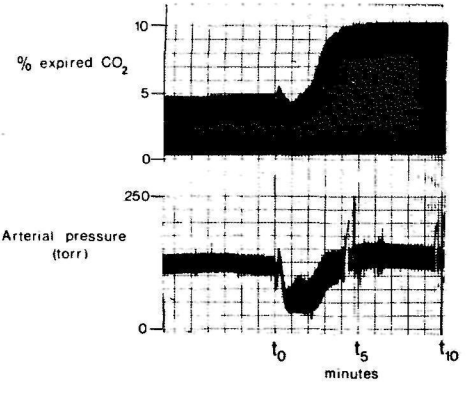

Fig. 3. Registration (original) of the effect of the administration of succinylcholine 2 mg/kg bw plus halothane 3% v/v on the end-expiratory CO_2, starting from t_0. At t_5 and t_{10} arterial blood samples are drawn, as can be seen from the interruption of the continuous pressure registration.

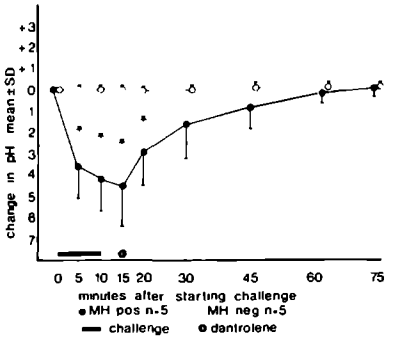

Fig 4 Change in arterial pH from the prechallenge value on exposure to the challenge of a bolus succinylcholine 2 mg/kg bw plus halothane 3% v/v Administration of dantrolene sodium 1 mg/kg bw at t15 concomitantly with hyperventilation F_{IO_2} 1 0 Statistically significant differences between groups * $P<0.05$

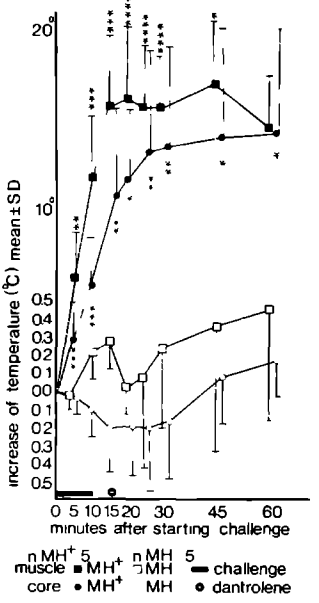

Fig 5 Change in temperature of skeletal muscle (m biceps femoris) and core temperature The challenge is performed with succinyl choline 2 mg/kg bw plus halothane 3% v/v for 10 min Dantrolene sodium is administered at t15 1 mg/kg bw concomitantly with hyperventilation, F_{IO_2} 1 0 Statistically significant differences between groups Muscle
* $P<0.05$ ** $P<0.01$ *** $P<0.005$, **** $P<0.001$
Core ⊛ $P<0.05$, ⊛⊛ $P<0.01$ ⊛⊛⊛ $P<0.005$, ⊛⊛⊛⊛ $P<0.001$

Table 1

Muscle metabolites in skeletal muscle of MH+ (n=5) and MH pigs (n=5, measured in state A before challenge)

Parameter	MH+	MH	P value
ATP	6 0±0 9	4 6±0 8	0 030
ADP	0 4±0 1	0 2±0 1	0 023
AMP	0 2±0 1	0 3±0 2	NS
EC	0 94±0 01	0 91±0 06	NS
IMP	0 5±0 1	0 2±0 1	0 001
NAD+	0 8±0 1	0 8±0 3	NS
PCr	13 5±2 2	25 4±3 0	0 001
Cr	28 7±6 5	15 3±1 3*	0 011
Cr/Cr+PCr	0 677±0 033	0 359±0 010*	0 001
Lactate	23 9±4 3	6 3±1 2	0 001
PCr/lactate	0 57±0 06	3 96±0 93	0 001
Pi	9 3±2 3	8 5±1 6	NS

Metabolite concentrations are expressed as nmol mg wet weight ±standard deviation NS=not significant
EC=Energy Charge (ATP+1/2 ADP) / (ATP+ADP+AMP)
* n=3
MH+=malignant hyperthermia susceptible MH =controls
ATP=adenosine triphosphate ADP=adenosine diphosphate AMP=adenosine monophosphate IMP=inosine monophosphate, NAD+=nicotinamide adenine dinucleotide Cr=creatine PCr=phosphocreatine Pi=inorganic phosphate

However, muscle temperature increased slightly during the challenge (0 2°C) and dropped after the administration of dantrolene (Fig 5)

2 Biochemical data

The levels of the muscle metabolites in state A of both the MH+ pigs as well as the MH pigs are shown in Table 1 Significant differences between the MH+ pigs and the MH pigs were found an increased level of ATP, ADP, IMP, Cr, lactate and increased ratio Cr/(Cr+PCr) was measured in the MH+ pigs compared to the MH- pigs A decreased level was measured for PCr and the ratio PCr/lactate comparing MH+ pigs with MH- pigs The ranges of the metabolites in state A which differ with the highest degree of significance are shown in Table 2 No

Table 2

Ranges of muscle metabolites levels in skeletal muscle of MH+ (n=5) and MH pigs (n=5 measured in state A before challenge) Only those values are shown with $P<0.011$

Parameter	MH+	MH
IMP	0 4-0 6	0 0-0 3
PCr	11 1-17 0	21 7-26 9
Cr	23 6-39 7	13 8-16 1
Lactate	20 4-30 9	4 4-7 8
PCr/lactate	0 53-0 67	2 78-5 32
Cr/Cr+PCr	0 64 0 72	0 35-0 37

Concentrations are expressed as nmol/mg wet weight (For abbreviations, see Table 1)

Table 3
Muscle metabolites in skeletal muscle of MH^+ pigs (n=5)

Parameter	State A	State B	State C	*P* value comparing A/B	A/C	B/C
ATP	6 0±0 9	3 3±1 4	3 3±1 9	0 030	NS	NS
Pi	9 3±2 3	12 5±3 1	8 3±0 8	0 041	NS	NS
PCr	13 5±2 2	4 0±2 3	12 5±7 1	0 001	NS	NS
ADP	0 4±0 1	0 5±0 3	0 3±0 1	NS	NS	NS
Lactate	23 9±4 3	30 0±14 1	10 6±4 5	NS	0 015	0 039
IMP	0 5±0 1	1 1±0 5	0 6±0 3	NS	NS	NS
AMP	0 2±0 1	0 2±0 1	0 2±0 1	NS	NS	NS
NAD^+	0 8±0 1	0 6±0 2	0 5±0 2	NS	0 035	NS
EC	0 94±0 01	0 89±0 03	0 89±0 05	0 011	NS	NS

Metabolite concentrations are expressed as nmol/mg wet weight ±standard deviation NS=not significant
EC=Energy Charge (ATP+1/2 ADP) / ATP+ADP+AMP)
State A before challenge
State B after 10 min challenge with halothane 3% and a bolus injection of succinylcholine 2 mg/kg bw
State C 30 min after the administration of dantrolene sodium 1 mg/kg bw
P values are determined according to the 2 tailed Student s *t* test for paired values
p A/B=*P* value comparing state A to state B id
(For abbreviations see Table 1)

overlap was seen in the measured values when comparing the MH^+ pigs to the MH^- pigs

The effects of the challenge and the treatment with dantrolene on the muscle metabolites of the MH^+ pigs are shown in Table 3 A significant decrease occurred in ATP and PCr levels and the energy charge (EC), comparing state A to state B The administration of dantrolene produced a significant decrease in lactate, comparing state C to both state B and state A

The effects of the challenge and the administration of dantrolene on the muscle metabolites of the MH pigs are shown in Table 4 Comparing state A to C and state B to C, no significant changes were evident with respect to any muscle metabolite levels

Table 4
Muscle metabolites in skeletal muscle of MH pigs (n=5)

Parameter	State A	State B	State C
ATP	4 6±0 8	3 3±0 9	4 3±1 1
Pi	8 5±1 6	9 9±3 8	8 5±2 2
PCr	25 4±3 0	15 7±8 1	22 8±2 2
ADP	0 2±0 1	0 2±0 1	0 2±0 0
Lactate	6 3±1 2	10 0±8 7	5 5±1 9
IMP	0 2±0 1	0 5±0 3	0 3±0 1
AMP	0 3±0 2	0 2±0 2	0 3±0 3
NAD^+	0 8±0 3	0 6±0 2	0 6±0 1
EC	0 91±0 06	0 92±0 03	0 91±0 08

Metabolite concentrations are expressed as nmol/mg wet weight ±standard deviation
EC=Energy Charge (ATP+1/2 ADP) / ATP+ADP+AMP)
State A before challenge
State B after 10 min challenge with halothane 3% and a bolus injection of succinylcholine 2 mg/kg bw
State C 30 min after the administration of dantrolene sodium, 1 mg/kg bw
(For abbreviations, see Table 1)

The activities of the muscle enzymes adenylate kinase and AMP-deaminase are shown in Table 5 No differences were demonstrated in activity of adenylate kinase comparing state A to B, B to C and A to C, in the MH^+ pigs or in the MH pigs Comparing the adenylate kinase activity between the MH^- pigs and the MH^+ pigs, no difference was found Concerning the activity of AMP-deaminase, no difference was measured between the MH^+ pigs and the MH^- pigs comparing state A to B, A to C and B to C

3 Serological results

In the MH^+ pigs the challenge caused an increase of the levels of lactate, K^+, Pi as well as CK and LDH After the administration of dantrolene sodium, an elevation of these parameters was still seen In the MH^- pigs no changes occurred in the investigated parameters

4 Results of the in vitro muscle contraction test

The *in vitro* muscle test confirmed the increased susceptibility to caffeine and caffeine+halothane of the MH^+ pigs' skeletal muscle On exposure to 4 mM and 8 mM caffeine, all muscle samples from the MH^+ pigs reacted with a contracture However, in the controls, one sample also produced a contracture on exposure to 4 mM caffeine All samples from the controls reacted with a contracture on exposure to caffeine 8 mM

The combination of 1% halothane plus 0 5 mM or 1 mM caffeine did not produce a contracture in the muscle

Table 5
Muscle enzyme activities of MH^+ (n=4) and MH^- pigs (n=4)

	MH^+ pigs			MH^- pigs		
	State A	State B	State C	State A	State B	State C
Adenylate kinase	324±34	382±38	394±98	322±16	317±35	342±24
AMP deaminase	53±20	49±25	63±25	63±24	55±10	60±7

Enzyme activities are expressed as nmol product formed/mg protein hour Mean values±s d are given
State A before challenge
State B after 10 min challenge with halothane 3% and a bolus injection of succinylcholine 2 mg/kg bw
State C 30 min after the administration of dantrolene sodium 1 mg/kg bw

samples taken from the MH^- pigs On exposure to 0 5 mM caffeine plus 1% halothane, muscle samples taken from 3 MH^+ pigs reacted with a contracture On exposure to 1 mM caffeine plus 1% halothane, samples from 4 MH^+ pigs reacted with a contracture Muscle samples from one MH^+ pig did not react at all This was presumably due to the almost complete loss of energy-rich phosphates, which was shown by the analysis of this muscle sample

DISCUSSION

The present results illustrate a number of important physiological changes during an MH crisis Especially the immediate increase of expiratory CO_2 after the start of the challenge is a characteristic finding This increased CO_2 output (measured with a capnograph) enables the anaesthetist to diagnose an MH crisis in the very early phase of this life-threatening condition The only known condition producing a comparable increase of CO_2 output during anaesthesia is a thyroid storm Another symptom of MH, increase of body temperature, is a relatively late symptom as can be concluded from the temperature registrations (Fig 5) The blood gas analyses during the MH crisis show a significant increase of $Paco_2$ and a decreased base excess Furthermore, an increased concentration of lactic acid is found in the muscle of the MH-susceptible pigs as compared to the controls This means that the changes in acid-base status during an MH period are of both aerobic and anaerobic muscular origin, as has also recently been shown by Hall et al (13)

This increased metabolism during an MH period is not able to supply sufficient amounts of energy-rich phosphates, as can be concluded from the decrease in ATP, PCr and EC values during the MH crisis Furthermore, an increased concentration of Pi is measured during the MH crisis This means that during the MH crisis, hydrolysis of energy rich phosphates occurs

The reduced concentration of both ATP and PCr in the skeletal muscle of the MH^+ pigs during the MH crisis may be caused either by a reduced production or by an increased consumption of energy-rich compounds, or a combination of these two factors

An increased consumption occurs during muscle contraction, caused by the activation of the actinomyosine complex by calcium Evidence of a reduced capacity for synthesis of ATP by mitochondria isolated from the MH-susceptible pigs during the MH crisis is presented in the following paper (3) This means that during an MH crisis, both an increased consumption of energy-rich phosphates and a possibly reduced production are present The discussion on mitochondrial metabolism in the second paper of this series (3) is also relevant Ultimately, this will lead to loss of cellular integrity due to loss of ATP, needed for ATP-dependent reactions like the electrochemical gradient over the cellular membranes A reduction of the concentration of ATP in human muscle has been ascribed to a deficiency of muscle adenylate kinase (14) This enzyme catalyses the formation of 1 mol ATP and 1 mol AMP out of 2 mol ADP However, in our experiments no significant differences were found between the adenylate kinase activity in the MH^+ pigs, comparing state A to B No significant difference was found comparing the enzyme activity in the MH^+ pigs to the MH^- pigs, both in state A, B and C This means that the enzyme adenylate-kinase is not deficient in MH^+ pigs, either pre-existent or induced The same conclusion has been drawn from other studies of adenylate kinase activities in MH susceptible pigs (15) and MH susceptible humans (16)

Another enzyme involved in the regulation of ATP levels is AMP-deaminase This enzyme stabilises the EC by reducing the concentration of AMP This is achieved by deamination of AMP leading to the formation of IMP and NH_3 However, no significant differences were found in the activity of this enzyme in the MH^+ pigs, comparing state A to state B or state C Also, no significant difference was measured, comparing the MH^+ pigs to the MH pigs, in state A, B and C So,

neither the enzyme adenylate kinase nor the enzyme AMP-deaminase seems to be involved in the metabolic changes in the MH crisis in pig skeletal muscle.

The high lactate concentration in the skeletal muscle of the MH^+ pigs in state A may point to a pre-existent derangement of cellular homeostasis in muscle metabolism, as has also been shown in MH-susceptible humans (17). Moreover, the administration of pentobarbital will increase the concentration of PCr in skeletal muscle (18). So, the observation of an increased lactate concentration as well as a decreased PCr concentration in skeletal muscle of the MH^+ pigs, as compared to the MH^- pigs, points to the involvement of the skeletal muscle in the pathogenesis of MH

Whether these observations of a reduced concentration of PCr and an increased concentration of lactate are due to the stress of the experiment or to an expression of a pre-existent condition, cannot be decided at present. However, we may exclude the former as all animals received premedication of fentanyl and droperidol, which are known to delay stress-induced MH in pigs (19). Furthermore, all first muscle samples were taken at least 60 min after induction of anaesthesia, during a stable phase of the experimental procedure.

The effect of the administration of dantrolene is markedly expressed by the decrease of the lactate concentration in the skeletal muscle and a reduction of $Paco_2$ and an increase in arterial pH. This reduction of lactate concentration influenced by the administration of dantrolene may point indirectly to the underlying triggering mechanism of MH Dantrolene is known to reduce the intracellular concentration of calcium in frog muscle fibre (20). A role of calcium in the triggering of MH has been suggested (21) No pertinent proof has yet been provided with respect to increased calcium concentrations in the MH skeletal muscle during an MH crisis. It is known that calcium is able to initiate several biochemical processes For example, calcium activates the enzyme phosphorylase, leading to the breakdown of glycogen Furthermore, an increased concentration of myoplasmic calcium initiates muscle contraction. These two processes consume ATP. The increased calcium and the increased Pi in the sarcoplasma are a well-known condition for the mitochondria specifically to accumulate calcium at the expense of the phosphorylation of ADP. Evidence for the accumulation of calcium in the mitochondria of the MH-susceptible pigs during the MH crisis is presented in the third paper of this series (4).

Concerning the *in vitro* muscle test, one false positive reaction was found in our MH^- pigs on exposure to 4 mM caffeine. However, on exposure to halothane 1% plus increasing concentrations of caffeine, it was possible to differentiate between the MH^- pigs and the MH^+ pigs.

The observation of an almost complete loss of energy-rich phosphates in one muscle sample from an MH susceptible pig points to the vulnerability of the *in vitro* test. Apparently, the removal and subsequent storage of this muscle sample caused the loss of biochemical compounds, leading to the loss of viability of the muscle specimen.

The conclusion which can be drawn from this part of our study is that it is of utmost importance to measure expiratory CO_2 concentration continuously, in order to make the diagnosis of MH in a very early phase of the MH attack. This statement is supported by a calculation of the carbon dioxide production based on the respiratory volume and $Paco_2$ in two MH cases in humans (22). The analysis of muscle samples, taken before the triggering of an MH attack, enables us to diagnose MH susceptibility on the metabolic profile in the porcine MH model. This method may also be useful in screening MH susceptibility in humans Furthermore, triggering of MH produces a highly significant drop in the concentration of PCr in the MH^+ muscle. The reduction of ATP is less significant than the reduction in PCr This observation suggests that the *in vitro* ATP depletion test (23) should be amended to include the PCr content in the muscle metabolite analysis.

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REFERENCES

1 Gronert G A Malignant hyperthermia *Anesthesiology* 1980 **53**: 395-423

2 Harrrison G G Porcine malignant hyperthermia In Britt B A, ed Malignant hyperthermia, *Int Anaesthesiol Clin* 1979 **17**: 25-61.

3 Ruitenbeek W, Verburg M P, Janssen A J M, Stadhouders A M, Sengers R C A *In vivo* induced malignant hyperthermia in pigs II metabolism of skeletal muscle mitochondria *Acta Anaesthesiol Scand* 1984 **28** 9-13

4 Stadhouders A M, Viering W A L, Verburg M P, Ruitenbeek W, Sengers R C A *In vivo* induced malignant hyperthermia in pigs:

III localization of calcium in skeletal muscle by means of electron microscopy and microprobe analysis *Acta Anaesthesiol Scand* 1984 **28** 14-26
5 Wick S M, Hepler P K Selective localization of intracellular Ca^{2+} with potassium antimonate *J Histochem Cytochem* 1982 **30**: 1190-1204
6 Eikelenboom G, Minkema D Prediction of pale, soft, exudative muscle with a non-lethal test for the halothane induced porcine malignant hyperthermia syndrome *Neth J Vet Sci* 1974 **99**: 421-426
7 Kalow W, Britt B A, Terreau M E, Haist C Metabolic error of muscle metabolism after recovery from malignant hyperthermia *Lancet* 1970 **ii**: 895-898
8 Ellis F R, Harriman D G F, Currie S, Cain P A Screening for malignant hyperthermia in susceptible patients In Aldrete J A, Britt B A, eds Second international symposium on malignant hyperthermia, New York Grune & Stratton 1978 273-285
9 Oerlemans F T J J, Van Bennekom C, De Bruyn C Metabolite profiling of human muscle extracts by isotachophoresis *J Inher Metab Dis* 1981 **4**: 109-110
10 Leech A R, Newsholme E A Radiochemical assays for adenylate kinase and AMP deaminase, using polyethyleneimine-cellulose thin layers. *Anal Biochem* 1978 **90**: 576-589
11 Lowry O H, Rosebrough N J, Farr A L, Randall R J Protein measurement with the folin phenol reagent *J Biol Chem* 1951 **193**: 265-275
12 Jaworek D, Gruger W, Bergmeyer H U In Bergmeyer ed Methoden der Enzymatischen Analyse II Weinheim Verlag Chemie, 1974 2178-2181
13 Hall G M, Lucke J N, Orchard C, Lovell R, Lister D Porcine malignant hyperthermia VIII leg metabolism *Br J Anaesth* 1982 **54** 941-947
14 Schmitt J, Schmidt K, Ritter H Hereditary malignant hyperpyrexia associated with muscle adenylate kinase deficiency *Hum Genet* 1974 **24**: 253-257
15 Marjanen L A, Denborough M A Adenylate kinase and malignant hyperpyrexia *Br J Anaesth* 1982 **54**: 949-952
16 Cerri C G, Willner J H, Britt B A, Wood D S Adenylate kinase deficiency and malignant hyperthermia *Hum Genet* 1981 **57**: 325-326
17 Isaacs H, Heffron J J A Morphological and biochemical defects in muscles of human carriers of the malignant hyperthermia syndrome *Br J Anaesth* 1975 **47**: 475-481
18 Heffron J J A, McLoughlin J V Influence of halothane, pentobarbital, carbon dioxide and ether anaesthesia on high energy phosphate levels in mouse and frog skeletal muscle *Gen Pharm* 1976 **7**: 63-65
19 McGrath C J, Rempel W E, Addis P B, Crimi A J Acepromazine and droperidol inhibition of halothane induced malignant hyperthermia (porcine stress syndrome) in swine *Am J Vet Res* 1981 **42**: 195-199
20 Hainaut K, Desmedt J E Effect of dantrolene sodium on calcium movements in single muscle fibres *Nature* 1974 **252**: 728-729
21 Britt B A Etiology and pathophysiology of malignant hyperthermia *Fed Proc* 1979 **38**: 44-48
22 Liebenschütz F, Mai C, Pickerodt V W A Increased carbon dioxide production in two patients with malignant hyperpyrexia and its control by dantrolene *Br J Anaesth* 1979 **51**: 899-903
23 Britt B A, Endrenyi L, Kalow W, Peters P L The adenosine triphosphate depletion test comparison with the caffeine contracture test as a method of diagnosing malignant hyperthermia susceptibility *Can Anaesth Soc J* 1976 **23** 624-635

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CHAPTER 6

In vivo induced malignant hyperthermia in pigs. II. Metabolism of skeletal muscle mitochondria.

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In Vivo Induced Malignant Hyperthermia in Pigs II. Metabolism of Skeletal Muscle Mitochondria

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The biochemical characteristics of skeletal muscle mitochondria of malignant hyperthermia (MH) susceptible Dutch Landrace pigs have been investigated before and during an MH attack, induced *in vivo* by halothane plus succinylcholine. The muscle homogenates have a decreased capacity to synthesize ATP and creatine phosphate during the MH period. Muscle mitochondria prepared from susceptible pigs in an MH period consume less oxygen than do mitochondria isolated before the attack, or mitochondria from control pigs during the challenge. The oxidative phosphorylation is not uncoupled during the critical period. The production of CO_2 indicates that the *in vitro* measured capacity of the MH muscle mitochondria correctly reflects the *in vivo* condition during the MH attack. The restricted synthesis may be caused by a factor, finding expression in the mitochondria themselves, and obtained or activated during the MH attack.

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Key words Energy-rich compounds; halothane, malignant hyperthermia, mitochondria, oxidative phosphorylation, pigs, respiratory chain, skeletal muscle

Many studies have been published concerning the pathological conditions leading to malignant hyperthermia (MH) in humans during anaesthesia with halothane and/or depolarizing muscle relaxants. Recently, research on human subjects as well as on pig models has been reviewed (1, 2). Many conflicting data about the cause and course of events during a hyperthermic attack have been reported. In many studies a limited number of *in vitro* effects has been examined, ignoring the importance of the (patho-)physiological role of the agents used. The pathology of MH is most expressed in skeletal muscle tissue.

We designed a study on a large number of, especially muscle-related, parameters measured before and during *in vivo* induced hyperthermic periods in Dutch Landrace pigs. In this way the extra-muscular contributions to the hyperthermic state, such as the influences of the nervous system, hormone-producing tissues, blood supply, liver and other organs are not left out of consideration. A few studies on MH induction in whole pigs have previously been published (3, 4, 5, 6), but they dealt mainly with a restricted number of either serological or muscle metabolite alterations.

In the preceding paper (7) the physiological investigations and muscle metabolite levels were described. In contrast to studies revealing *in vitro* halothane effects on isolated mitochondria, the present study is concerned with the biochemistry of muscle mitochondria from pigs in the MH period *in vivo*. Oxygen consumption has been measured in isolated muscle mitochondria, while the overall aerobic metabolism has been investigated by means of synthesis of adenosine triphosphate (ATP) and creatine phosphate in muscle homogenates as recently described (8).

The involvement of the mitochondria in calcium homeostasis is described in the accompanying paper (9).

MATERIALS AND METHODS

The descent, selection procedure, pretreatment and halothane challenge of the Dutch Landrace pigs have been reported in the preceding paper (7). Biopsies from the biceps femoris muscle, weighing about 2 g, were taken before (state A) and after 10 min of halothane plus succinylcholine challenge (state B). The temperature of the tissue fractions was kept between 0° and 4°C from the moment of biopsy until starting assays (within 2 h).

The capacity of the muscle to synthesize ATP and creatine phosphate was tested in a 600 g supernatant in the presence of pyruvate, malate, adenosine diphosphate (ADP) and creatine, as described previously (8). Incubation times were 0 and 60 min, while Ap_5A (P^1, P^5-di(adenosine-5^1-)pentaphosphate) was added to inhibit adenylate kinase activity (10). The incubation times for the estimation of the activity of adenylate kinase were 0 and 90 s, Ap_5A being omitted in these assays (8).

The isolation procedure for mitochondria and measurement of the oxygen consumption in the presence of pyruvate plus malate at a

temperature of 37°C has been published elsewhere (11, the only modification being that the final suspension still contains heparin) The concentrations of oligomycin and of FCCP (carbonyl cyanide p-trifluomethoxy-phenylhydrazone) were 20 and 1 μg per ml incubation medium, respectively.

The cytochrome contents of the mitochondria were estimated by means of the difference spectrum of the oxidized and reduced forms (with succinate plus cyanide) (12) Cytochrome oxidase activity was measured at 25°C according to Cooperstein & Lazorow (13)

Protein content was determined as described by Lowry et al (14) with bovine serum albumin as standard

During all the assays no halothane or any other hyperthermia-inducing agent was added

Student's *t*-test was used for statistical analysis

RESULTS

The synthesis of ATP plus creatine phosphate by muscle homogenates in the presence of Ap_5A is shown in Table 1. No statistically significant difference exists between the capacity of control and MH^+ pig muscle in state A to synthesize the high-energy compounds from pyruvate plus malate. However, the muscle of the MH^+ pigs can produce less ATP plus creatine phosphate than the control animals if the muscles are isolated during the hyperthermic period (state B).

Table 2 summarizes the oxygen consumption of the isolated mitochondria in the presence of pyruvate plus malate. In the presence of sufficient amounts of ADP and the other substrates, the mitochondria are in state 3, synthesizing ATP. After exhaustion of ADP the organelles reach state 4. The RCI (respiratory control index) is defined as the ratio of oxygen consumption in state 3 divided by that in state 4. The RCI is an index for the intactness of the mitochondria. injured membranes result in a low value for the RCI. The P/O-ratio (ADP phosphorylated/oxygen consumed) is a measure for the degree of coupling between the respiratory process and ADP phosphorylation. Tightly coupled mitochondria show the maximal, theoretical P/O-ratio of 3 in the case of pyruvate and malate as substrates. It is clear from Table 2 that the oxygen consumption in state 3 by mitochondria isolated from MH^+ pigs during the attack has declined as compared to mitochondria isolated in the pre-challenge phase. Halothane and succinylcholine administration to the control animals, however, has no effect on the activity of the respiratory chain of the muscle mitochondria. The addition of oligomycin (inhibitor of the oxidative phosphorylation process and of Mg^{2+}-ATPase) and of FCCP (uncoupler of the phosphorylation from the respiration process) to the mitochondria revealed no abnormalities in the MH^+ pigs. From the decreased oxygen consumption in state 3 and the normal P/O-ratio, it may be concluded that the mitochondria of MH^+ pigs can function only suboptimally during the hyperthermic period. This is in agreement with the incubation experiments with homogenates as summarized in Table 1. Normal levels of cytochrome aa_3, b and $c+c_1$ and normal cytochrome oxidase activity in the mitochondria of the MH^+ pigs have been found.

Incubation of the homogenate without the inhibitor of adenylate kinase gives an indication of the adenylate kinase activity in the homogenate (8). The ATP synthesized under these conditions is shown in Table 3. It is clear that the MH^+ animals are not deficient in adenylate kinase.

Table 1

ATP plus creatine phosphate synthesized by pig muscle homogenates (in mmol per min per kg protein) via oxidative phosphorylation of ADP, in the presence of Ap_5A (mean ± s d)

	MH^+ pigs (n=3)	Control pigs (n=4)
Before MH induction	75±30	96±33
During MH induction	67±15*	107±16

* Difference between MH^+ and control pigs $P<0.015$.
MH^+ = malignant hyperthermia susceptible

Table 2

Oxygen consumption by isolated pig muscle mitochondria (in mg-atom oxygen per min per kg protein) before and during MH challenge (mean ± s d)

	MH^+ pigs (n=3)		Control pigs (n=4)	
	Before	During	Before	During
State 3	337±42	188±68*§	317±26	308±29
State 4	75±13	50±13	63±32	54±25
State 4+oligomycin	70±17	36±12	48±22	43±15
State 4+FCCP	255±29	224±53	311±31	218±101
RCI	4 5±0 3	3 9±1 2	5 9±2 4	6 8±3 3
P/O-ratio	2 8±0.1	2 6±0 1	2 8±0 2	2 8±0 2

* Difference between MH^+ and control value $P<0.025$
§ Difference between before and during induction $P<0.03$
MH^+ = malignant hyperthermia susceptible
RCI = respiratory control index
P/O-ratio = ADP phosphorylated/oxygen consumed
FCCP = carbonyl cyanide p-trifluomethoxy-phenylhydrazone

Table 3

ATP synthesis in pig muscle homogenates (in mmol per min per kg protein) by the adenylate kinase reaction (mean ± s.d.).

	MH^+ pigs (n=3)	Control pigs (n=4)
Before MH induction	1247±361*	811±39
During MH induction	1201±301*	804±68

* Difference between MH^+ and control pigs $P<0.02$
MH^+ = malignant hyperthermia susceptible

DISCUSSION

The respiratory chain of mitochondria from MH^+ animals has been reported to possess decreased, normal and even increased activity (for review, see Gronert (2)) We have tested the mitochondrial energy supply in two different ways in muscles of Dutch Landrace pigs The investigations on both production of high energy compounds and oxygen consumption reveal that during MH induction the MH^+ muscles have only about 60% of the capacity of the control muscles to synthesize ATP by means of oxidative phosphorylation (Tables 1 and 2) At first sight, the limited respiration may seem to be a direct consequence of the presence of halothane in the mitochondria Halothane has been reported to inhibit the electron flux through the NADH-Coenzyme Q-complex of the respiratory chain (15–21) However, the effect of halothane on the respiration in isolated MH^+ mitochondria is identical to its effect in MH^- mitochondria (20, 21) The present study does not reveal an inhibition of oxidative phosphorylation in MH^- mitochondria in state B As there is no reason to suppose a lower concentration of halothane in MH^- mitochondria as compared with MH^+ mitochondria, halothane *per se* is probably not the inhibiting factor of respiration in MH^+ muscle A further argument against an *in vitro* inhibition by halothane is that halothane probably has already evaporated during the preparative steps (16) It is calcium, free or bound to membranes, that may be considered as the inhibiting factor Its level is greatly enhanced in MH^+ mitochondria in state B (9) Furthermore, the MH^+ mitochondria reveal an increased sensitivity to exogenous calcium (22) The mechanism of the calcium influence is still obscure A calcium binding site in the NADH-Coenzyme Q-complex may play a role in the calcium and/or halothane interactions (23) The localization of a defect at that site links up with the normal levels of cytochromes we have found in MH^+ muscle Unfortunately, no studies have been published, in which the effect of halothane in MH^+ mitochondria has been compared with its effect in MH^- mitochondria, both in the presence of calcium

Another calcium-sensitive process is the mitochondrial ATP-ADP translocation The exchange of ATP and ADP through the mitochondrial inner membrane can be inhibited by intramitochondrial calcium ions (24) A partial dysfunctioning of the transport system during the challenge as a consequence of calcium accumulation cannot be ruled out

The production of ATP by MH^+ muscle mitochondria during the MH attack is probably much lower *in vivo* than suggested by the *in vitro* measured capacities of Tables 1 and 2 After 10 min of challenge the mitochondria have accumulated much calcium (9) As mitochondria prefer to employ their energy to take up calcium ions to phosphorylating ADP (22), most probably the *in vivo* ATP production by mitochondria is very low during this phase (The measurements on the isolated mitochondria could not have been disturbed by the presence of calcium ions in the incubation medium, as in that case an elevated oxygen consumption and a very low P/O-ratio would have been found (19, 25, 26))

One can calculate, using the *in vitro* measured capacity of the respiratory chain of Table 1 and stoichiometry for the ATP synthesis from pyruvate of 12 1 for muscle supernatants, that the volume of CO_2 produced by the MH^+ muscles during the challenge amounts to about 0 7 l min^{-1} $animal^{-1}$ The mean volume of CO_2 expired by MH^+ pigs during that period amounts to about 0 8 l min^{-1} $animal^{-1}$ (7) It can be concluded that the *in vitro* measured capacity approaches the *in vivo* activity of the respiratory chain very well The *in vivo* activity is probably the net effect of a stimulation by sarcoplasmic calcium ions of the oxygen consumption to a higher rate than ADP can achieve (25, 26), and an inhibiting effect of the lowered pH (27, 28) as a consequence of the production of lactate and CO_2 (7)

The low creatine phosphate level during the attack in MH^+ pigs (7) may be the result of an increased demand for energy, and a low rate of ADP phosphorylation caused by an increased level of sarcoplasmic calcium The conversion of ATP into creatine phosphate by the creatine kinase reaction, however, is not disturbed, either before or during the challenge, as the value of the mass action ratio for this reaction, i e $(CrP \times ADP)/(Cr \times ATP)$ is not different from the control group Its value, between $3\,4 \times 10^{-5}$ and $8\,3 \times 10^{-5}$, is identical to the value found for human muscle (unpublished observations) The most obvious cause of the low level of creatine phosphate in MH^+ muscle in the pre-challenge period (7) is the elevated lactate level, already present in the basal state (7) Harris et al (29) reported an inverse relationship between the lactate and creatine phosphate concentrations in human muscles The underlying mechanism may be a lowering of the muscle pH by lactic acid, driving the creatine kinase reaction in the direction of free creatine (30) Leakage of creatine out of the pathological muscles can be excluded, as the sum of free and phosphorylated creatine did not decline (7)

During the first years of MH research, it was supposed that the characteristic liberation of an abundance of heat during the attack was caused by uncoupled mitochondria, but afterwards this explanation seemed difficult to maintain (for review, see ref 2) Table 2 clearly shows that the muscle mitochondria of MH^+ pigs synthesize a sufficient amount of ATP per unit of oxygen. So, uncoupling of the bulk of the muscle

mitochondria cannot be the cause of the pathological thermogenesis during the first 10 min of the challenge. Berman et al. (3) indirectly came to the same conclusion, but they stated that the heat production in pigs could also not be ascribed to (normally coupled) mitochondrial activity However, using the data of Table 2 and the data of Wang et al. (31), one can calculate that coupled MH^+ muscle mitochondria can produce a temperature increase of the muscular tissue of one degree within 5-10 min. This value conforms to the observed temperature increase (7). So, the present study shows that the heat generation in MH^+ pig muscle can be ascribed for the major part to respiratory activity of coupled mitochondria. A largely aerobic origin of the heat generation in pigs during the first stages of MH was also stated by Hall et al. (32) Of course, heat production by neutralization of protons and hydrolysis of the high-energetic phosphate bond of ATP molecules, used in ion transport processes and contraction, will also occur. As long as it is hard to explain the amount of evolved heat during simple muscle contractions under experimental conditions (33), one cannot expect an accurate interpretation of the heat production in MH (3, 31).

At present, it is impracticable to schematize exactly the series of processes resulting in an MH attack From the present series of studies it may be clear that the muscle mitochondria play an important role, principally functioning in the removal of calcium from the sarcoplasm The ATP required has mainly been produced by glycolysis, but the consumption of ATP exceeds its production (7). A quantitatively important role for alternative metabolic pathways, for example, the ω-oxidation of fatty acids instead of β-oxidation, has been ruled out, because no other organic acids than lactate could be detected in the serum of the MH^+ (and MH^-) swine during the halothane challenge (unpublished observations). This finding is in agreement with the data of Berman et al. (3). It is clear from the experiments, summarized in Table 3 that the MH^+ animals are not deficient as to muscular adenylate kinase activity A more direct estimation of this activity leads to the same conclusion (7). Previously, an adenylate kinase deficiency has been reported in human MH (34), and such a deficiency has even been blamed for the energy crisis in the MH syndrome (35). However, recently a report was published which shows normal values for adenylate kinase in the examined families (36). The deficiency is likely not essential for the development of an MH attack.

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REFERENCES

1 Britt B A Etiology and pathophysiology of malignant hyperthermia *Fed Proc* 1979 **38**: 44-48
2 Gronert G A Malignant hyperthermia. *Anesthesiology* 1980 **53**: 395-423
3 Berman M C, Harrison G G, Bull A B, Kench J E Changes underlying halothane-induced malignant hyperpyrexia in Landrace pigs. *Nature* 1970 **225**: 653-655
4 Lucke J N, Hall G M, Lister D Porcine malignant hyperthermia I Metabolic and physiological changes *Br J Anaesth* 1976 **48**: 297-304
5 Gronert G A, Theye R A Halothane-induced porcine malignant hyperthermia metabolic and hemodynamic changes *Anesthesiology* 1976 **44**: 36-43
6 Mitchell G, Heffron J J A, van Rensburg A J J A halothane-induced biochemical defect in muscle of normal and malignant hyperthermia-susceptible Landrace pigs *Anesth Analg* 1980 **59**: 250-256
7 Verburg M P, Oerlemans F T J J, van Bennekom C A et al *In vivo* induced malignant hyperthermia in pigs I Physiological and biochemical changes and the influence of dantrolene sodium *Acta Anaesthesiol Scand* 1984 **28** 1-8
8 Ruitenbeek W, Sengers R C A, Trijbels J M F, Stadhouders A M, Janssen A J M Estimation of energy metabolism in human skeletal muscle homogenate as a diagnostic aid *J Inher Metab Dis* 1981 **4**: 91-92
9 Stadhouders A M, Viering W A L, Verburg M P, Ruitenbeek W, Sengers R C A *In vivo* induced malignant hyperthermia in pigs III Localization of calcium in skeletal muscle mitochondria by means of electron microscopy and microprobe analysis *Acta Anaesthesiol Scand* 1984 **28** 14-26
10 Kuby S A, Hamada M, Gerber D et al Studies on adenosine triphosphate transphosphorylases, isolation and several properties of the crystalline calf ATP-AMP transphosphorylases (adenylate kinases) from muscle and liver and some observations on the rabbit muscle adenylate kinase *Arch Biochem Biophys* 1978 **187**: 34-52
11 Bookelman H, Trijbels J M F, Sengers R C A, Janssen A J M, Veerkamp J H, Stadhouders A M Pyruvate oxidation in rat and human skeletal muscle mitochondria *Biochem Med* 1978 **20**: 395-403
12 Bookelman H, Trijbels J M F, Sengers R C A, Janssen A J M Measurement of cytochromes in human skeletal muscle mitochondria, isolated from fresh and frozen stored muscle specimens *Biochem Med* 1978 **19**: 366-373
13 Cooperstein S J, Lazorow A A microspectrophotometric method for determination of cytochrome oxidase *J Biol Chem* 1951 **189**: 665-670
14 Lowry O H, Rosebrough N J, Farr A L, Randall R J Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951 **193**: 265-275
15 Miller R N, Hunter F E Jr The effect of halothane on electron transport, oxidative phophorylation, and swelling in rat liver mitochondria *Mol Pharmacol* 1970 **6**: 67-77
16 Harris R A, Munroe J, Farmer B, Kim K C, Jenkins P Action of halothane upon mitochondrial respiration. *Arch Biochem Biophys* 1971. **142**: 435-444

17 Eikelenboom G Stress-susceptibility in swine and its relationship with energy metabolism in skeletal musculature Thesis, University of Utrecht, The Netherlands, 1972
18 Cohen P J Effect of anesthetics on mitochondrial function *Anesthesiology* 1973 **39:** 153–164
19 Rosenberg H, Haugaard N The effects of halothane on metabolism and calcium uptake in mitochondria of the rat liver and brain *Anesthesiology* 1973 **39:** 44–53
20 Britt B A, Endrenyi L, Cadman D L, Fan H M, Fung H Y-K Porcine malignant hyperthermia effects of halothane on mitochondrial respiration and calcium accumulation *Anesthesiology* 1975 **42:** 292–300
21 Gronert G A, Heffron J J A Skeletal muscle mitochondria in porcine malignant hyperthermia respiratory activity, calcium functions, and depression by halothane *Anesth Analg* 1979 **58:** 76–81
22 Cheah K S, Cheah A M Mitochondrial calcium transport and calcium activated phospholipase in porcine malignant hyperthermia *Biochim Biophys Acta* 1981 **634:** 70–84
23 Grist E M Baum H A possible mechanism for the halothane-induced inhibition of mitochondrial respiration binding of endogenous calcium to NADH dehydrogenase *FEBS Lett* 1974 **48:** 41–44
24 Gomez-Puyou A, Tuena de Gomez-Puyou M, Klapp M, Carafoli E The effect of calcium on the translocation of adenine nucleotides in rat liver mitochondria *Arch Biochem Biophys* 1979 **194** 399–404
25 Lehninger A L, Carafoli E, Rossi C S Energy-linked ion movements in mitochondrial systems *Adv Enzymol* 1967 **29:** 259–320
26 Carafoli E, Lehninger A L A survey of the interaction of calcium ions with mitochondria from different tissues and species *Biochem J* 1971 **122:** 681–690
27 Mitchelson K R, Hird F J R Effect of pH and halothane on muscle and liver mitochondria *Am J Physiol* 1973 **225:** 1393–1398
28 Schaffer S W, Safer B, Ford C, Illingworth J, Williamson J R Respiratory acidosis and its reversibility in perfused rat heart regulation of citric acid cycle activity *Am J Physiol* 1978 **234:** H40–H51
29 Harris R C, Sahlin K, Hultman E Phosphagen and lactate contents of m quadriceps femoris of man after exercise *J Appl Physiol* 1977 **43:** 852–857
30 Sahlin K, Harris R C, Hultman E Creatine kinase equilibrium and lactate content compared with muscle pH in tissue samples obtained after isometric exercise *Biochem J* 1975 **152:** 173–180
31 Wang J K, Moffitt E A, Rosevaer J W Oxidative phosphorylation in acute hyperthermia *Anesthesiology* 1969 **30:** 439–442
32 Hall G M, Bendall J R, Lucke J N, Lister D Porcine malignant hyperthermia II Heat production *Br J Anaesth* 1976 **48:** 305–308
33 Curtin N A, Woledge R C Energy changes and muscular contraction *Physiol Rev* 1978 **58:** 690–761
34 Schmitt J, Schmidt K, Ritter H Hereditary malignant hyperpyrexia associated with muscle adenylate kinase deficiency *Hum Genet* 1974 **24:** 253–257
35 Junger H, Kuhnle P, Schorer R Neue Aspekte zum Krankheitsbild der malignen Hyperthermie und seine Therapie *Z Kinderchir* 1980 **30:** 35–37
36 Cerri C G, Willner J H, Britt B A, Wood D S Adenylate kinase deficiency and malignant hyperthermia *Hum Genet* 1981 **57:** 325–326

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CHAPTER 7

In vivo induced malignant hyperthermia in pigs. III. Localization of calcium ions in skeletal muscle by means of electronmicroscopy and microprobe analysis.

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In Vivo Induced Malignant Hyperthermia in Pigs. III. Localization of Calcium in Skeletal Muscle Mitochondria by Means of Electronmicroscopy and Microprobe Analysis

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Biceps femoris muscle biopsies of malignant hyperthermia susceptible (MH^+) and non-susceptible (MH^-) Dutch Landrace pigs were studied ultrastructurally, and exchangeable calcium was demonstrated, using the antimonate precipitation technique in combination with electron probe x-ray microanalysis Biopsies were taken before and during the administration of halothane-plus-succinylcholine and after dantrolene sodium treatment of the animals MH^+ muscle, taken before the MH triggering, showed a high proportion (about 35%) of cells with supercontraction Both MH^+ and MH^- muscle had broad but nearly identical ranges of cell diameter Core-like structures were occasionally present in muscle from MH^+ pigs Muscle mitochondria from the MH^+ pigs accumulated large amounts of calcium in their matrix compartment during the halothane-plus-succinylcholine induced MH crisis This calcium loading in the course of time caused swelling and structural damage to the mitochondria Skeletal muscle mitochondria from MH^- pigs did not show such a reaction pattern on challenge with halothane and succinylcholine It is concluded that in MH^+ pigs the challenge brings about an increase in myoplasmic free calcium, which is predominantly due to calcium influx from the extracellular fluid This rise in cytosolic calcium causes the mitochondria to accumulate the cation in an energy-dependent way These findings are discussed in relation to the diverging halothane and caffeine contraction responses of aerobic type I and anaerobic type II muscle fibres

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Key words Calcium, electron microprobe, electron microscopy, halothane, malignant hyperthermia, mitochondria, pigs, pyroantimonate precipitation, skeletal muscle, succinylcholine

Malignant hyperthermia (MH) is a serious pharmacogenetic syndrome that can be induced in predisposed individuals by volatile anaesthetics or depolarizing muscle relaxants; notorious agents being the widely used anaesthetic halothane and the muscle relaxant succinylcholine (1, 2).

Investigations on MH-susceptible pigs have been particularly helpful in characterizing the physiological and metabolic de-arrangements that occur during the course of an MH crisis (3, 4, 5). Although there is now compelling evidence that the skeletal muscle tissue is the primary target tissue (6, 7), the intracellular mechanisms responsible for the series of biochemical events leading to the syndrome are as yet poorly understood (8).

A widely accepted theory states that halothane causes MH-susceptible muscle to lose its ability to regulate the calcium distribution properly over the various cellular organelles and to develop an excess of calcium ions in the myoplasm (9, 10). It is assumed that the increased myoplasmic free calcium triggers the MH-associated rigidity as well as the increased metabolic activity (11, 12, 13).

Two mechanisms for the increase of the calcium concentration in the myoplasm may be involved. One is the release of calcium from the sarcoplasmic reticulum (SR), the other is a direct inflow of calcium from the extracellular fluid into the myoplasm, where the calcium concentration is some orders of magnitude higher than in the myoplasm (14).

Recently, it has been suggested that the primary defect which initiates MH may lie in the plasma membrane of the skeletal muscle cells (15, 16). Gallant and coworkers (15) found that halothane, at clinical concentrations, produced a depolarization of susceptible skeletal muscle, but did not affect the resting membrane potential of skeletal muscle from normal pigs. Such a

depolarization might result in the release of a small amount of "triggering calcium", followed by the release of more calcium from the SR or by an increased calcium inflow from the extracellular fluid (16). The studies reported here were undertaken in order to trace subcellular calcium movements in skeletal muscle fibres after an *in vivo* induced MH crisis in MH-susceptible pigs. For this purpose, use was made of the antimonate precipitation technique (17, 18), in combination with electron probe x-ray microanalysis of the deposits.

MATERIAL AND METHODS

Animals

Ten 3-6 month-old pigs, five malignant hyperthermia-susceptible (MH^+) and five control (MH^-) Dutch Landrace swine were used for these studies The pigs were classified according to their reaction to halothane anaesthesia at 6 weeks of age by the method of Eikelenboom & Minkema (19) The anaesthetic techniques have been described in the accompanying paper by Verburg and co-workers (20)

Histological techniques

Biopsies from the biceps femoris muscle were taken immediately before the halothane-succinylcholine administration (state A), at the end of a 10-min challenge period (state B) and 30 min after administration of dantrolene sodium (state C) that was given 15 min after starting the challenge For further details, see accompanying paper (20) Before dissecting the muscle strips from the muscle bulk, they were maintained at constant longitudinal tension by clamping them *in situ* in special muscle biopsy clamps

For routine microscopic studies, the strips were immersed in a fixative, consisting of 2% glutaraldehyde in 0 1 M cacodylate buffer, to which 4% sucrose was added (pH 7 4, 550 mOsm, 20-22°C) After 1 h, the tissue was carefully freed from adhering connective tissue and diced into cubes of approximately 1 mm^3 Fixation was continued for a total period of 24 h Postfixation was in 2% OsO_4 in 0 1 M cacodylate buffer for 2 h The specimens were then washed, dehydrated and embedded in Epon 812 resin For light microscopy, 0 5 µm sections were stained with toluidine blue (1%) in sodiumborate (1%) or with paraphenylenediamine (1%) For electron microscopy, thin sections were double-stained with uranyl acetate and lead citrate according to standard procedures

Calcium localization

For selective localization of intracellular calcium, the antimonate precipitation technique was used in conjunction with elemental analysis of the precipitate by electron probe x-ray microanalysis (21, 22) The muscle biopsies were treated with potassium pyroantimonate (KPA) essentially according to the prescriptions of Schafer (22) Muscle tissue cubes of about 0 5 mm^3 were fixed for 4 h in a freshly prepared 2% (w/v) KPA-solution (Merck, Darmstadt, Germany) in 3% (v/v) purified glutaraldehyde, brought to pH 7 8 with a trace of 0 01 N NaOH or 0 01 N acetic acid Thereafter, the specimens were washed briefly in a freshly prepared solution of KPA (2% w/v) in distilled water Postfixation was in a 2% OsO_4-solution, also containing 2% KPA. The tissues were washed, dehydrated and embedded in epon

The elemental composition of the electron dense antimonate precipitates was studied for the presence of Ca, Na and K by electron probe x-ray microanalysis Use was made of a Cameca model Camebax-MBS 70 microprobe, equipped with a Cameca universal wavelength dispersive WD x-ray spectrometer as well as a Microtrace 30 sq mm energy dispersive (ED) lithium-drifted silicon x-ray detector For peak identification and data processing a Tracor TN 2000 and TN 1310 x-ray microanalysis system was used

Tissue sections of 100-300 nm thickness were collected on copper or nickel grids, carbon-coated and studied with or without counterstaining Analyses were performed at 30 kV accelerating voltage with beam currents of 26-35 nA and a spot diameter of 0 3-0 5 µm

The Lα-radiation was used for identification of antimony (effective Lα-energy 3 604 keV) and osmium (effective Lα-energy 8 904 keV), see ED-spectrum (Fig 1a) The lighter elements were identified by their Kα-radiation (sodium-Kα 1 041 keV, potassium effective Kα 3 312 keV, calcium Kα 3 690 keV) The problem of overlap in ED spectrometry between the Lα-emission of Sb and the Kα-emission of Ca was circumvented by using WD analysis only for the demonstration of the presence of calcium in the antimonate reaction products

Calcium was concluded to be present when in a scan around the characteristic wavelength (see Fig 1b) a 'significant' peak P was found, i e more than 3× the standard deviation above background B, $P\text{-}B \geq 3\times\sqrt{P+B}$ (23) Bulk standards of antimony, osmium, calcium

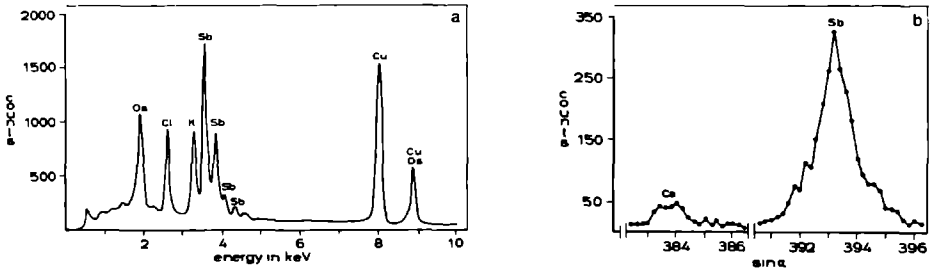


Fig 1(a) Example of an energy dispersive (ED) x-ray microanalysis spectrum of the antimonate precipitates in a mitochondrion (MH^+ muscle, state B) The osmium, chlorine, antimony and copper peaks are indicated Due to overlap with the antimony Lα peak, the calcium Kα peak (3 690 keV) cannot be discerned in this spectrum (b) Example of a wavelength dispersive (WD) analysis of antimonate precipitates in a mitochondrion (MH^+ muscle, state B) The calcium Kα peak (sinα=0 3839) can readily be discerned from the antimony $L\alpha_1$ peak (sinα=0 39335) The calcium peak is 'significant', i e $P\text{-}B \geq 3\times\sqrt{P+B}$ (P=0 005)

and potassium were used for spectrometer calibration Absolute quantitation of elemental concentrations was not pursued The sensitivity of x-ray analysis of our probe for the ions studies is approximately 10^{-15}-10^{-17} g per unit area probed (21)

RESULTS

I. *Histopathological abnormalities of MH⁺ muscle*

The results to be described here concern the MH⁺ animals; only when appropriate will the findings in the MH⁻ animals also be reported.

State A. Light microscopic investigation of semi-thin longitudinal sections revealed a high incidence of fibres with areas of considerable supercontraction: (about 35%, Fig. 2a) Irregular dark staining fibre zones were alternating with lighter areas of stretched sarcomeres. This phenomenon more frequently occurs in the fibres with relatively small numbers of mitochondria (presumed white or intermediate). Such aberrant fibres were also noticed in the muscular tissue of MH⁻-animals, although their number was far higher in the MH⁺ pigs.

Another prominent finding in the MH⁺ muscular tissue was a marked variation in fibre sizes (see Fig. 2b).

The distribution of the small fibres over the muscle fascicles tended to be random, but occasionally an area of seemingly grouped atrophy was noticed. Neither small angulated fibres nor 'porcine skeletal muscle giant fibres' were seen (24). Histographic analysis of the fibre diameters revealed a unimodal frequency distribution with diameters ranging from less than 10 to 80 μm (mean 43.0 μm, s d. 13.5). However, the MH⁻ pigs showed an almost identical frequency distribution of fibre diameters (mean 41.0 μm, s.d. 15.6), (see Fig. 3).

Hyaline degeneration and necrosis of thin fibres was very rare; infiltration of activated histiocytes and fibroblasts was not observed. With some variation from animal to animal, about 18% of the fibres had internal nuclei (MH⁻ animals: less than 3%).

Structurally altered fibres such as target or core fibres, were not evident from semi-thin resin sections. In the electron microscope, however, evidence of myofibrillar disruption, not related to the phenomenon of supercontraction or overstretching of sarcomeres was noticed in about 3% of the fibre (see Fig. 2e). In the affected fibre areas the normal banding pattern was absent and the myofilaments showed marked malalignment, making the areas resemble 'unstructured' cores The transition from the core to the non-core area, however, was not sharply delimited, whereas mitochondria were not always lacking in the affected fibre zones.

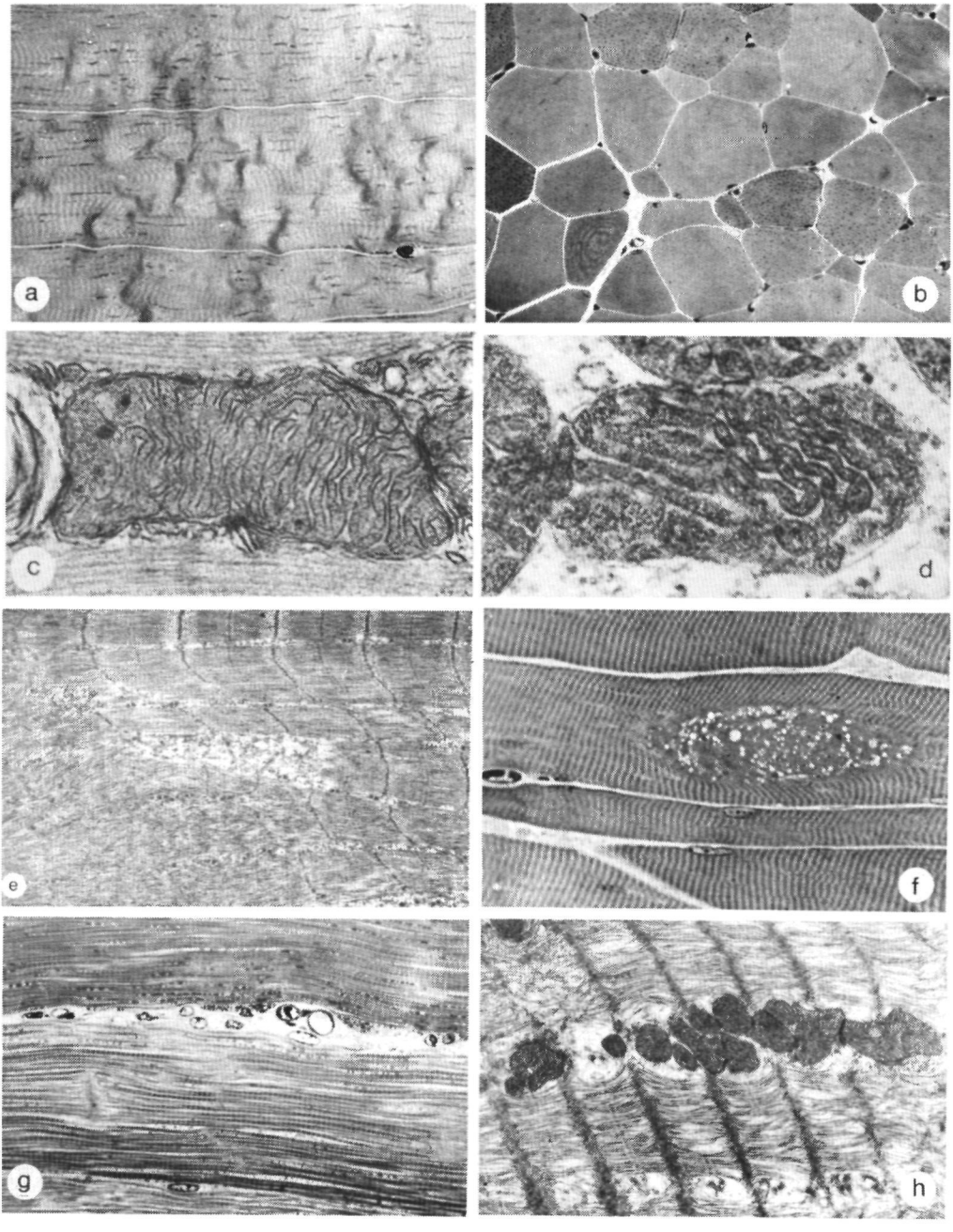
The mitochondria in the muscle fibres characteristically had an angulated 'zig-zag' appearance of their cristae (Fig. 2c). The branching and anastomosing cristae frequently formed a complex honeycomb pattern of membranes enclosing cylindrical or circular compartments of the mitochondrial matrix (Fig. 2d). Sometimes considerable subsarcolemmal aggregation of mitochondria was present. Otherwise, electron microscopy revealed no significant structural abnormalities of these organelles; in particular, no mitochondria with crystalloid inclusions in the intracristal spaces were seen. Glycogen particles appeared quite abundant in many muscle fibres.

State B. The histological appearance of the muscle tissue from MH⁺ pigs taken during state B differed from that of the pre-challenge condition in that fibres with areas of supercontraction and overstretching of sarcomeres were only incidentally seen. On the other hand, in many fibres there was a narrowing and sometimes complete disappearance of the I-zones with accordingly an overall shortening of the sarcomere length, indicating that these fibres were in a contracted state. Moreover, the myofibrils in many fibres were widely spaced, giving the fibres an oedematous appearance (see Fig. 2g). Mitochondria were seen as small rounded particles in these dilated intermyofibrillar crevices.

Electron microscopy revealed that in most fibres the myofibrils themselves had a normal-looking appearance with no apparent abnormalities primarily affecting the actin and myosin filaments. In the widened intermyofibrillar spaces elements of the T-tubule system and sarcoplasmic reticulum were present, but glycogen particles were very much reduced. Only occasionally were fibres encountered with supercontracted sarcomeres and serious disruption and loss of myofilaments (Fig. 2h).

Fig 2 Micrographs from porcine MH-susceptible biceps femoris muscle, Figs a-e, before halothane-succinylcholine challenge, state A, Figs f-h, 10 min after onset of MH crisis, state B (a) Longitudinally sectioned muscle fascicle, showing marked supercontraction and overstretching (Magn 770×) (b) Transversely sectioned muscle fascicle, showing marked variation in fibre diameter (Magn 610×). (c) Mitochondrion with a dense matrix and sharply angulating 'zig-zag' cristae (Magn 59,000×) (d) The cristae tend to fuse and to form tubule-like matrix compartments (Magn 73,000×) (e) Central region of myofibre with serious disorganisation of myofibrils and absence of banding pattern (Magn 18,000×). (f) Vacuolated area in muscle fibre core region, during MH crisis (Magn 1,325×) (g) Oedematous myofibres with separation of myofibrils and sarcolemmal aggregation of mitochondria (Magn 525×) (h) Cluster of mitochondria in a myofibre with strongly contracted sarcomeres and loss of myofilaments (Magn 17,300×)

Most of the mitochondria also had a normal-looking appearance with a moderately electron dense matrix and 'zig-zag' coursing cristae. But scattered between them markedly swollen mitochondria were seen with



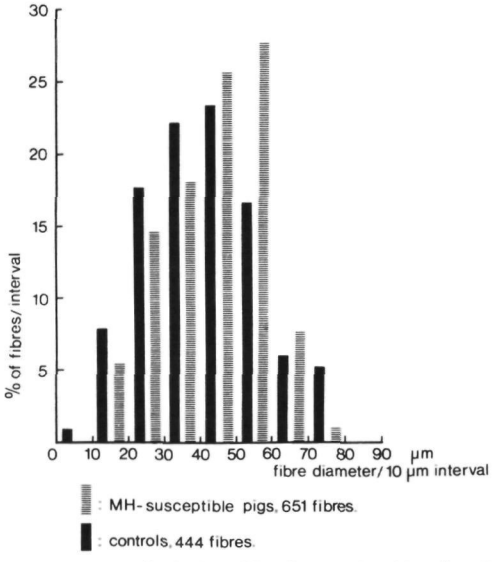


Fig. 3. Frequency distribution of fibre diameters from biceps femoris muscle in MH-susceptible and MH control pigs; as measured on 1 µm sections of epon-embedded tissue.

disoriented cristae and a patchy-appearing matrix. In a limited number of fibres (<1%), areas completely devoid of cross-striation and with a vacuolated appearance located in the core region of the fibres, were seen (Fig. 2f). Electron microscopy revealed that part of these vacuoles originated from strongly swollen mitochondria, but other vacuoles were single membrane bounded and contained finely granular material.

State C. The biopsies from the dantrolene-treated MH^+ animals showed a moderately oedematous muscular tissue, as judged by the extent of the intermyofibrillar spaces. Mitochondria in these fibres were round and often considerably swollen with an electron transparent matrix. The conformation of the cristae had changed from the angulated 'zig-zag' pattern to an unfolded parallel state. Many muscle fibres again had considerable contents of glycogen particles and hardly any fibre had areas of supercontraction or overstretching. A small percentage (3–5%) of fibres, however, showed areas with severely overcontracted degenerative myofilaments, forming true contraction clumps. In these partially necrotic fibres the plasmalemma mostly appeared focally disrupted and displaced from the basement membrane.

II. Calcium localization

In the biopsies of all three stages (A, B and C) of MH^+ and MH^- animals treated with KPA for the localization of calcium, considerable antimonate precipitation had occurred in the connective tissue sheaths around the muscle fibres. Especially the basement membrane of the muscle fibres and the capillaries reacted strongly and showed many antimonate deposits of varying sizes. The delicate network of fibrous tissue between the muscle fibres usually carried a scattered, randomly distributed fine precipitate.

When the electron-opaque precipitates in the endomysial or perimysial area were analysed with the electron probe, strong peaks for antimony (and osmium) as well as 'significant' peaks for potassium were consistently found. Although there was invariably a distinct indication for the presence of calcium and sodium in the skeletal muscle supporting tissue, significant peaks for these cations could only incidentally be registered.

Precipitate granules of varying sizes were always located in the fibrils and nuclei of muscle fibres. The distribution pattern of the precipitates in the myofibrils showed considerable variation from biopsy to biopsy and within one biopsy from fibre to fibre. A particular pattern of precipitate deposition, in some way or another related to the degree of contraction of the sarcomeres, could not be established. Generally speaking, the I-bands showed higher accumulations of antimony precipitates than did the A-bands, but these precipitates were only incidentally localized in transverse lines near the A-I junction.

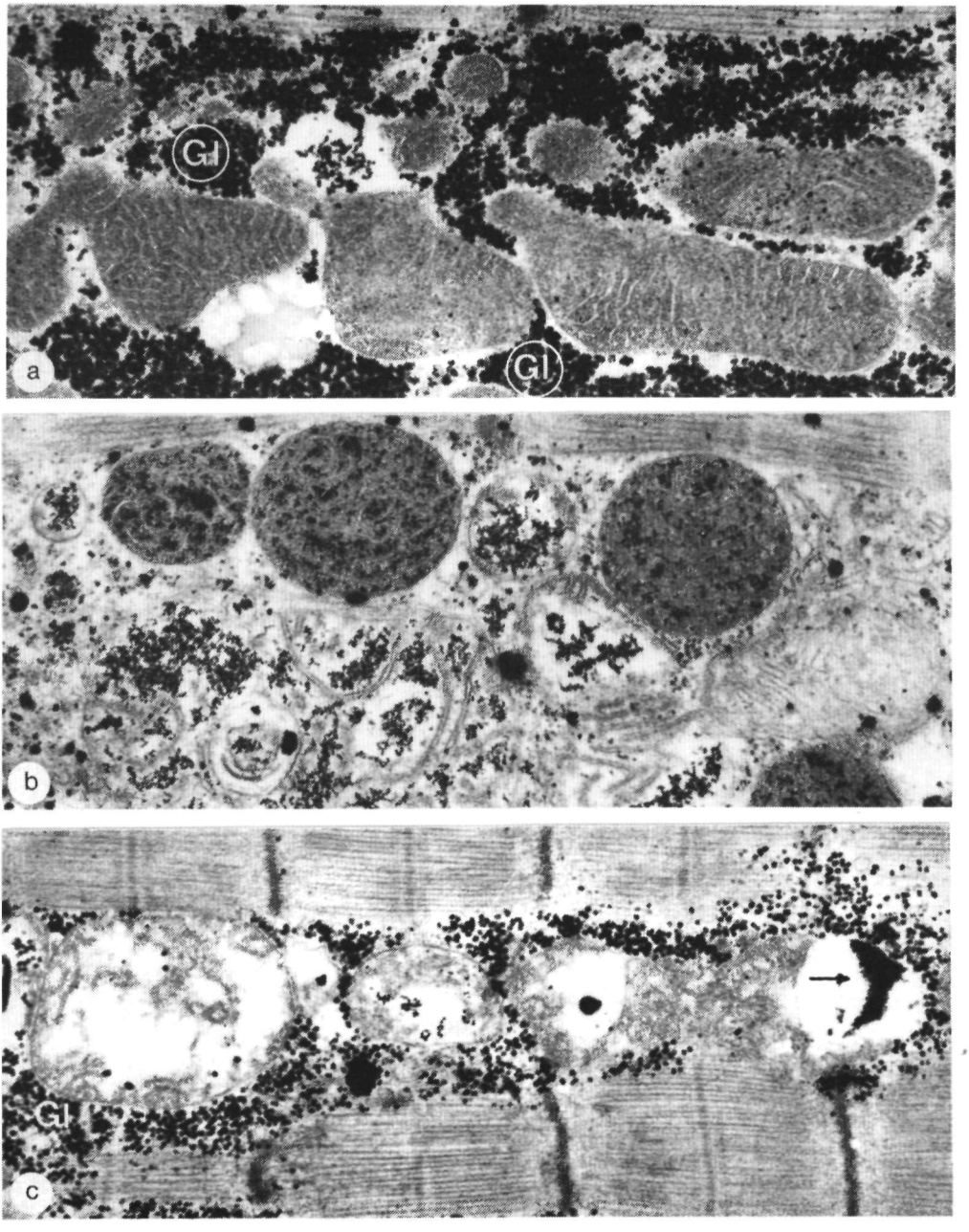
Because of this divergency in the pattern of pyroantimonate deposition in the myofibrils, no systematic microprobe studies of the cations precipitated in the sarcomeres have been performed. From the analyses that have been carried out, it appeared that significant calcium peaks could not be registered but that potassium was predominantly bound to the antimony.

In the following description of the KPA reactivity of the muscle fibres during state A, B and C, attention will

Fig. 4. Porcine MH^+ biceps femoris muscle, treated with antimonate for calcium localization. Biopsies were taken before (state A) and during a halothane + succinylcholine-induced MH crisis (state B), as well as 30 min after administration of dantrolene (state C). State A mitochondria (Fig. 4a) are virtually free from precipitate granules, but in state B the mitochondria are heavily loaded and show varying degrees of matrical swelling (Fig. 4b). In state C the mitochondria show massive enlargement of the matrix compartment, flocculent matrix protein densities and paucity of cristae (Fig. 4c). Glycogen granules (Gl), which strongly reacted with antimonate, are present in state A and state C, but are absent in state B. Fig. 4a, Magn.: 46,000×, Fig. 4b, Magn.: 54,000×, Fig. 4c, Magn.: 28,000×.)

be focussed on the mitochondria and the glycogen particles.

State A. In all tissue sections from MH^+ and MH^- animals treated with pyroantimonate, there was a strong KPA-



staining of the glycogen particles (see Fig. 4a). Granules in the subsarcolemmal and juxtanuclear areas, as well as those present in between the myofibrils and myofilaments all showed a strong KPA-reactivity. In fact, the KPA technique appeared quite effective in the localization of glycogen, allowing an easy distinction between glycogen-rich and glycogen-poor fibres. X-ray analysis of the glycogen deposits revealed that the main

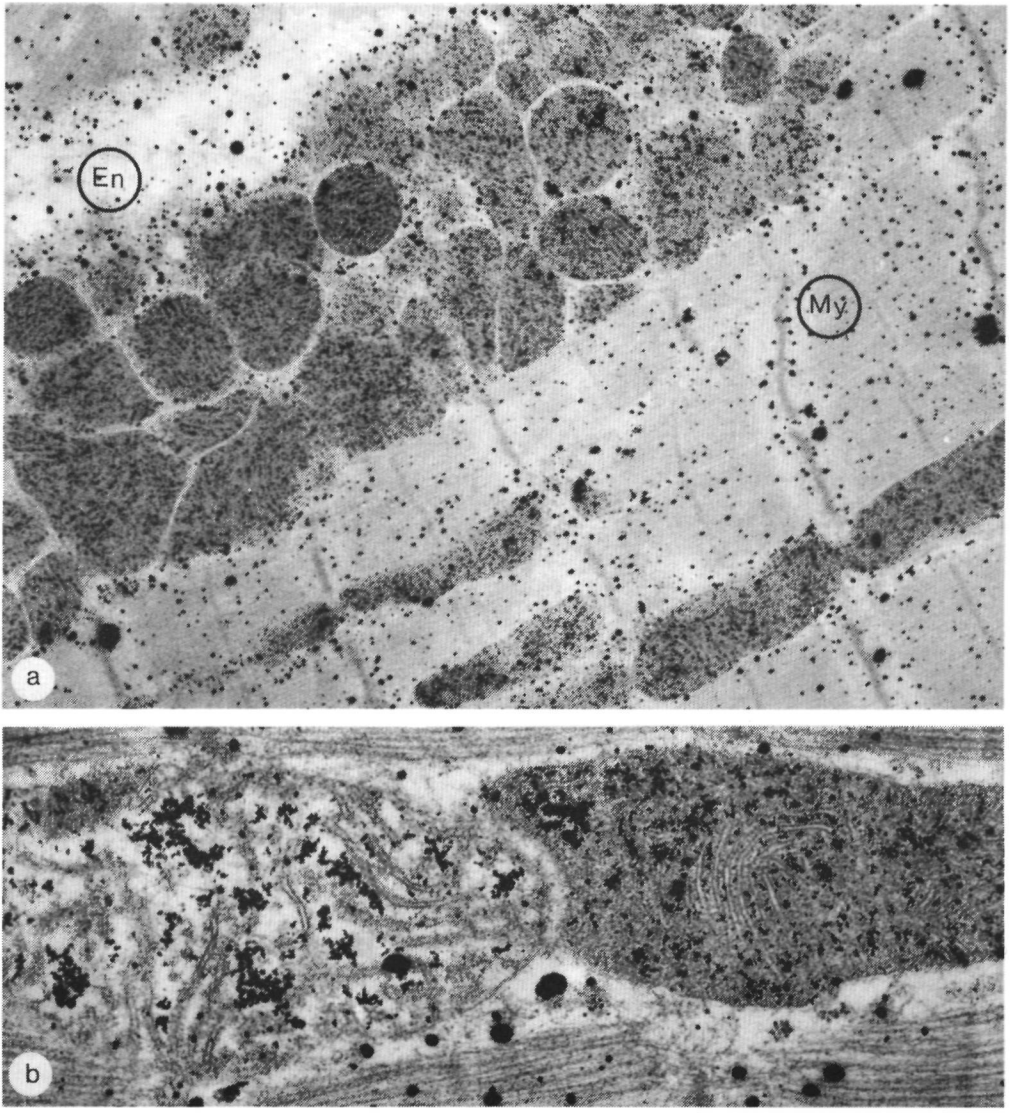


Fig. 5. Porcine MH$^+$ biceps femoris muscle, treated with antimonate for calcium localization. Biopsy taken during halothane-succinylcholine challenge (state B). (a) Subsarcolemmal and intermyofibrillar mitochondria contain numerous fine precipitation granules. Coarse granules are present in the endomysial (En) and myofibrillar (My) areas. (Magn.: 22,500×). (b) Two adjoining mitochondria, showing a distinctly different matrix condition. Note that antimonate precipitate granules do not occur in the intracristal spaces. (Magn.: 49,500×).

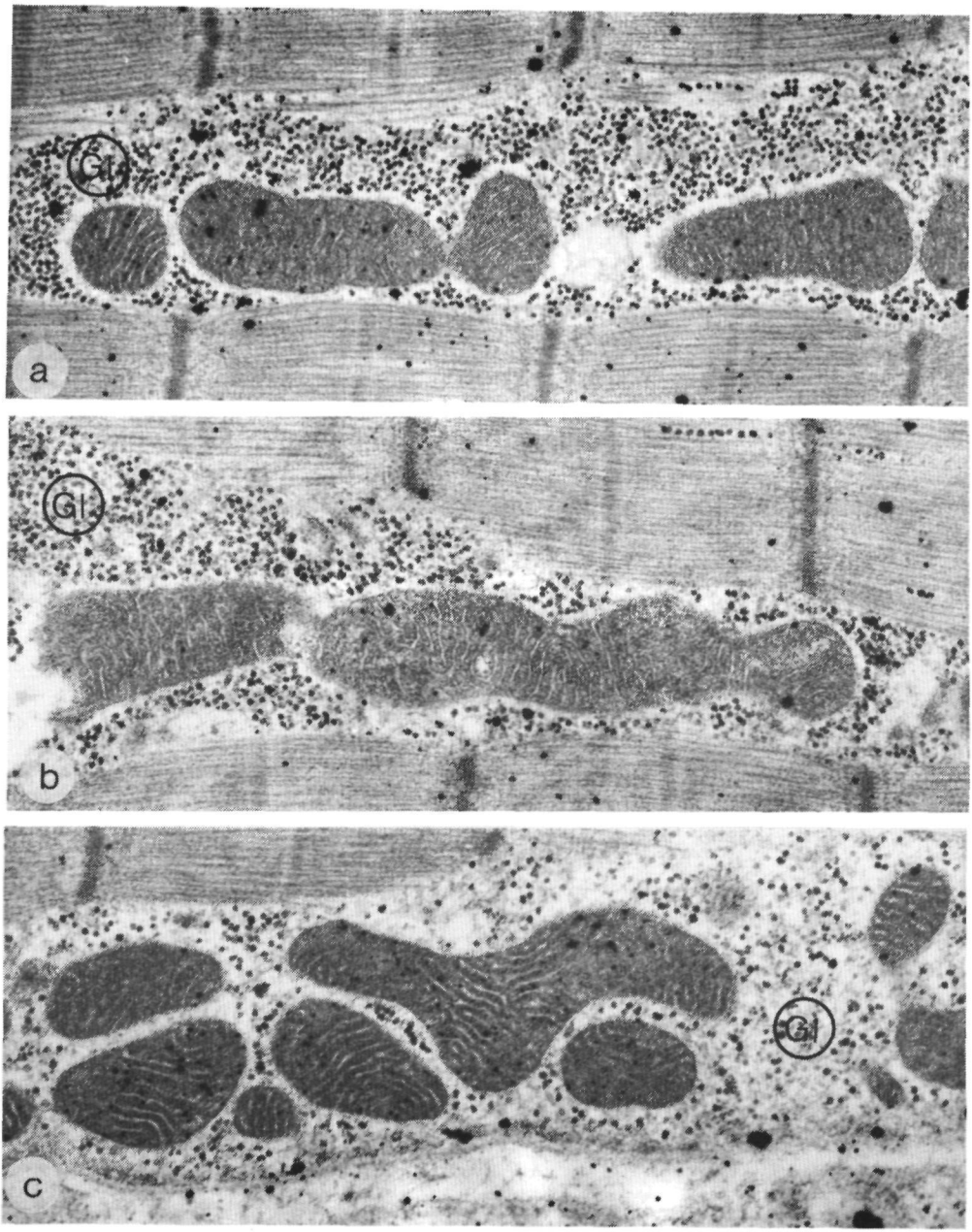


Fig. 6. Porcine MH⁻ biceps femoris muscle, treated with antimonate for calcium localization. Biopsies taken before (Fig. 6a) and during (Fig. 6b) a MH-inducing challenge and 30 min after administration of dantrolene (Fig. 6c). Considerable amounts of heavily staining glycogen particles are present in all three stages. The mitochondria contain small numbers of precipitate granules. (Fig. 6a, Magn.: 36,000×, Fig. 6b, Magn.: 41,000×, Fig. 6c, Magn.: 41,500×).

cation precipitated was potassium Significant peaks for calcium could only incidentally be obtained from glycogen areas In view of the size of the electron probe used for the x-ray generation we cannot definitely rule out whether in these cases some calcium-containing SR-elements were also hit by the electron probe

Mitochondria, either present in a strongly staining field of glycogen deposition or lying outside the glycogen areas (Fig 4a), were invariably completely free from antimonate granules or merely contained some fine non-aggregated precipitate in the condensed mitochondrial matrix

Analysis of the mitochondria did not produce sufficient calcium Kα counts to allow us to infer from these data that precipitation of calcium together with antimonate had occurred

State B The pattern of pyroantimonate deposition in MH^+ pigs during state B differed very markedly in two respects from the situation in susceptible pigs in state A

Firstly, in many - but not all - muscle fibres, the glycogen particles had almost completely disappeared (compare Fig 4a with Fig 4b) Second, in most of the muscle fibres the mitochondria showed large amounts of antimony precipitate (see Figs 4b, 5a and 5b) Indeed, on most occasions the organelles were strongly blackened by the abundance of electron-opaque deposits The precipitates were exclusively present in the matrix compartment of the mitochondria in the intracristal spaces and in the outer mitochondrial compartments antimonate precipitates were never encountered The mitochondrial reaction product was present as a finely granular non-aggregated precipitate, lying in rows between adjoining cristae free in the matrix or associated with the matrix face of the cristal membranes (Fig 4b, 5a and b) Sometimes, however, the precipitate granules tended to converge and this was invariably associated with swelling of the mitochondrial matrix (cf Fig 4b and 5b)

Mitochondria lying in close proximity usually showed about the same density of their precipitate content (Fig 5a) However, heavily loaded and less heavily loaded organelles (Fig 4b) as well as condensed and swollen mitochondria (Fig 5b) were regularly lying side by side, indicating that the organelles to a certain degree behaved according to an individual scheme

When occasionally in a particular muscle fibre the mitochondria had little or no antimony deposits, glycogen granules were present in fairly large amounts

Electron probe analysis of the animony precipitate-loaded mitochondria always yielded significant peaks for calcium (and most of the time also for potassium)

KPA-treated muscle tissue from MH^- control pigs during state B did not differ noticeably from the MH^- state A situation In particular, the mitochondria were either completely free from precipitate or merely had some fine and widely scattered granules (compare Fig 6a with 6b)

State C KPA-treated biopsy tissue from MH^+ pigs taken after dantrolene administration (state C) showed many muscle fibres in which positively stained glycogen granules were quite abundantly present (see Fig 4c) Very commonly, the mitochondria in these fibres showed distinct swelling of their matrix compartment (see Fig 4c) This applied not only to the peripheral mitochondria but also to the more deeply situated organelles In largely swollen mitochondria, the antimonate deposits had converged to some dense aggregates of finely granular precipitate per mitochondrial profile, which sharply contrasted with the electron-lucent matrix (see Fig 4c, arrow) Occasionally, glycogen granules were noticed to be present in the swollen mitochondrial matrix Electron probe analysis of these MH^+ state C mitochondria revealed no unequivocal indications for the presence of calcium in the organelles, i e 'significant' peaks could not be registered KPA-treated muscular tissue from MH^- pigs during state C showed a staining pattern that was quite comparable to that of the control state A and state B specimens, (compare Fig 6c with 6a and 6b)

DISCUSSION

The reported results point to a striking difference in the response of *in situ* skeletal muscle mitochondria from MH^+ and MH^- pigs when the animals are subjected to a succinylcholine-halothane challenge These results have been obtained using the antimonate precipitation procedure, which was originally introduced by Komnick (25) for the visualization of cations at the electron microscopical level It is now known that the pyroantimonate molecule is capable of forming precipitates with a number of inorganic cations, including those of sodium, potassium, calcium and magnesium (26, 27) In addition, it has been found that the KPA-technique under certain conditions is quite effective in the staining of particulate glycogen (28)

It is apparent from the literature that the results obtainable with the KPA-technique should be interpreted with caution, particularly with regard to the cation specificity of the pyroantimonate reaction (26) Many workers have attempted to introduce some means of discrimination between the cations precipitated For example, prior treatment of the tissue with calcium specific chelators (EGTA) has frequently been used to

demonstrate the presence of calcium in the antimonate reaction products The most appropriate way to study the presence of specific inorganic cations in the precipitates, however, seems to be the use of electron probe x-ray microanalysis (21, 22) This technique allows the fine structural localization of the precipitate to be directly related to accurate and sensitive elemental analysis Moreover, the relative concentrations of the cations precipitated can readily be determined It is of considerable advantage - as in our case - when both ED and WD x-ray spectrometry can be performed, because then problems of overlap between peaks lying near to each other, as for example the Sb-Lα and the Ca-Kα, can simply be circumvented

A considerable number of combined antimonate precipitation-x-ray microanalysis studies have been performed on normal and abnormal muscular tissue (21, 29, 30) It has become evident from these reports that the pyroantimonate particularly combines with calcium under the prevailing conditions of cation concentrations in muscle cells *In vitro* experiments also demonstrated that the sensitivity of the pyroantimonate ion for calcium precipitate formation is much greater than for other cations (27) Moreover, as a consequence of the difference between calcium and sodium in K-shell fluorescence yield and characteristic x-ray energy (31), the 'electron probe sensitivity' for calcium determination is at least one order of magnitude higher than for sodium Therefore, we consider that the antimonate precipitation technique is particularly appropriate for providing evidence about the location and translocation of exchangeable cellular calcium This is in agreement with Wick & Hepler (32), who in a recent review concluded that "antimonate is a valuable probe for localizing that subset of total Ca^{2+} which is loosely bound to cellular components and which therefore might be mobilized to effect regulation of various processes "

Our results with this technique demonstrate that the mitochondria in MH^{+} skeletal muscle fibres in response to the induction of an MH crisis start to accumulate calcium This mitochondrial calcium uptake occurs concomitantly with the disappearance of particulate glycogen granules Within the time of duration of the challenge (10 min), the calcium loading process induces considerable swelling of the matrix compartment in part of the mitochondria In view of the extensiveness of the mitochondrial calcium loading (cf Fig 5a) and in view of the ease with which significant calcium x-ray signals could be obtained from these mitochondria, the conclusion is inevitable that this calcium loading not only indicates a redistribution of exchangeable calcium within the muscle fibres, but also demonstrates that an influx of calcium from the extracellular spaces must have occurred These findings seem to agree well with the widely held view that the immediate cause of the acute metabolic crisis in MH is a sudden rise in the concentration of myoplasmic calcium (11)

To explain our findings, we postulate that as a direct response to the halothane+succinylcholine challenge an increase of the myoplasmic free calcium level occurs through influx from the extracellular fluid The mitochondria, in view of their exceedingly high affinity for this cation, start to sequester the calcium ions from their ambient medium In addition, the increased myoplasmic free calcium levels accelerate glycogenolysis because calcium induces activation of phosphorylase and this causes the rapid and complete disappearance of the glycogen particles

The remarkable calcium transporting capacities of mitochondria have been thoroughly studied in the past 15 years For details, the reader is referred to recent reviews (33, 34, 35) Because separate pathways for uptake and release have been found, a mitochondrial 'calcium cycle' has been proposed (36) It has been suggested that this calcium cycle plays the role of a versatile regulation mechanism for the distribution of cellular calcium (33, 34) This concept allows mitochondria to play an active role in calcium sequestration when cytosolic concentrations exceed a certain physiological level, about 1 μM according to (37) It also allows in theory for the control of intramitochondrial calcium, the presumed need for which comes from recent work on the calcium dependent activation of three intramitochondrial dehydrogenases (38), suggesting that calcium is potentially an important stimulator of the supply of reducing equivalents

In isolated mitochondria and in all probability also under *in vivo* conditions, the process of mitochondrial calcium accumulation is an energy-consuming respiration-dependent process that replaces and thus takes primacy over oxidative phosphorylation (39) The remarkable mitochondrial calcium storing activities in susceptible pigs during state B can therefore be taken to reflect the strong *in vivo* respiring activity of the calcium accumulating mitochondria This is in accordance with out biochemical findings (cf discussion of 2nd article in this series), (40)

About the actual submitochondrial compartmental distribution of calcium, little is known Recently, Happel & Simson (41) utilized the pyroantimonate precipitation method to localize calcium in isolated rat liver mitochondria in dependency of the metabolic state They found that the suborganellar localization varied in dependency of the metabolic state of the mitochondria Without succinate, mitochondria exhibited precipitates

primarily in the intracristal space In the presence of succinate, however, the localization was shifted towards the matrix compartment (with many of the densities associated with the matrix face of the cristal membranes)

The matrix localization of precipitates was especially obvious when calcium was present in the incubation medium Our studies revealed that in the MH^+, state B mitochondria, there was matrix antimony deposition only This finding presumably not only reflects the strong *in vivo* respiring activity of the calcium accumulating mitochondria, but also supports the well-documented evidence for a respiratory substrate-driven electrochemical potential in mitochondrial calcium transport (35, 39)

Evidence suggesting that the interaction of calcium with mitochondria plays an important role in the pathogenesis of the porcine MH syndrome has recently been given by Cheah & Cheah (42, 43, 44) These authors found that isolated MH^+ muscle mitochondria possessed significantly higher amounts of endogenous calcium and also showed significantly faster endogenous calcium efflux rates than did MH^- mitochondria (42) The coupling integrity of the isolated mitochondria, however, was identical The higher rate of calcium efflux was postulated to be due to the presence of a mitochondrial phospholipase (42)

Later, the same authors demonstrated that at increased temperature (40°C) the mitochondria from MH^+ pigs were more easily uncoupled by calcium than were the MH mitochondria, whereas the uncoupled mitochondria showed large amplitude swelling (43) The calcium-induced uncoupling and the large amplitude swelling were prevented by phospholipase inhibitors Subsequently, it was found that MH^+ and MH^- mitochondria had similar amounts of endogenous mitochondrial phospholipase A_2, but that the total activity in MH^+ pigs was at least twice that of normal (44)

Our observation that part of the mitochondria during the MH crisis show loss of their condensed configuration, become swollen and lose most of their calcium precipitate, may well be due to an increased activity of their phospholipase A_2 This enzyme will form fatty acids and lyso derivates of the mitochondrial phospholipids, which will cause the mitochondria to undergo large amplitude swelling, accompanied simultaneously by a calcium efflux (44) However, the swelling of the mitochondria may also be directly caused by the increasing accumulation of calcium and inorganic phosphate (Pi) in the mitochondrial matrix (39)

To explain the enhanced release of calcium in the sarcoplasm of MH^+ muscle, Cheah & Cheah (45) speculate that fatty acids and lyso-phospholipids will destabilize the mitochondrial membranes, causing the mitochondria to swell and to show a faster than normal calcium release Moreover, the fatty acids liberated are considered to inactivate the calcium transport systems of the SR, causing the release of additional calcium into the sarcoplasm (44, 45)

In our opinion, however, the cause of the increased mitochondrial phospholipase activity in MH^+ pigs may well be due to an increased concentration of calcium in the sarcoplasm, already present due to the stress of the pre-slaughter period In the experimental procedures of Cheah & Cheah, the mitochondria were isolated immediately post mortem It is well known, however, that the stress of the pre-slaughter period is capable of initiating an MH crisis Since mitochondrial phospholipase A_2 can already be activated by low concentrations of calcium (46), their observations of the differences in the mitochondrial phospholipase A_2 may, in fact, be caused by an enhanced mitochondrial calcium content as a direct consequence of an enhanced myoplastic calcium level, rather than being at the onset of an enhanced release of calcium from the SR or the mitochondria into the sarcoplasm

In our study, the EM findings during the prechallenge period do not show a difference in the amount of pyroantimonate precipitate comparing MH^+ pigs to controls This may mean that due to the premedication and the barbiturate anaesthesia, in our experiments, in the prechallenge period hardly any difference exists in the mitochondrial calcium concentration between the MH^+ and MH pigs

Our observations of a strong mitochondrial calcium loading during the initial phases of an MH crisis may also be of importance in view of the question recently posed, whether MH is a feature of a specific myofibre type (47)

Indeed, experimental evidence has been gained that the histochemical type I (or red) myofibres seem to be more susceptible to the action of drugs such as halothane and caffeine than are the type II (white) fibres (48, 49) McLoughlin et al (49) studied the delay effect of neuroleptic drugs on the onset of a halothane-induced porcine MH crisis They found that the fall in the concentration of creatine phosphate and the rise in lactate was more marked in the predominantly red than in the predominantly white fibre areas of the MH^+ semitendinosus muscle Nelson & Schochet (47) showed that the porcine MH^- trapezius muscle with about equal proportions of type I and type II fibres, had greater abnormal *in vitro* contracture responses to halothane and to caffeine than had MH^+ gracilis muscle with 10% type I and 90% type II fibres However, these latter authors also found that porcine control trapezius muscle com-

pared to control gracilis muscle had a greater contracture response to caffeine. Comparable results were obtained by Brownell & Szabo (48) in rat muscles.

The fact that both rat muscles and control porcine muscles showed different reactivities of the type I and type II fibres argues against the supposition that MH susceptibility is a disease of a specific myofibre type. Nevertheless, it indicates that the diverging contracture response of type I and type II fibres is mainly biochemical rather than physiological in origin and possibly related to energy metabolism. One might speculate that - on halothane+succinylcholine induced influx of extracellular calcium into the myoplasm - the aerobic type I fibres will lose their high energy phosphates more rapidly than will the anaerobic (glycolytic) type II fibres. This will be so because the mitochondria, their main energy-producing system, give priority to calcium accumulation above ADP phosphorylation under these conditions (33, 34) Due to these circumstances, the type I fibres in particular will be deprived of the possibility to mobilize their energy-consuming calcium pumps in order to sequester the increased myoplasmic calcium into the SR-compartments or to extrude it again into the extracellular space. Due to this adenosine triphosphate (ATP) shortage, also the calcium-induced myofibrillar contracted will be sustained. On the other hand, in the anaerobic type II fibres, the excess calcium will stimulate glycogenolysis by activating phosphorylase kinase, which, in a relative sense, will favour the anaerobic energy-yielding reactions, producing lactate.

Summarizing our findings, the presence of increased amounts of calcium in the MH^+ myofibres during the MH crisis is shown by our electronmicroscopic and microprobe studies. The amount of calcium precipitated apparently is far more than the amount that can potentially be liberated from intracellular calcium stores in the mitochondria or the SR-cisternae. An influx of calcium from the extracellular fluid is therefore postulated. The increase of the myoplasmic calcium level is counteracted by the mitochondria, starting to accumulate the calcium in an energy-consuming process. This process of calcium loading will take primacy over the phosphorylation of ADP into ATP. The increased concentration of myoplasmic calcium also stimulates glycogenolysis by activating the phosphorylase, resulting in degradation of the glycogen, i.e. the disappearance of the glycogen particles.

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REFERENCES

1 Denborough M A, Lovell R R H Anaesthetic deaths in a family *Lancet* 1960 **ii** 45
2 Britt B A, Locker W G, Kalow W Hereditary aspects of malignant hyperthermia *Can Anaesth Soc J* 1969 **16** 89-98
3 Sybesma W, Eikelenboom G Malignant hyperthermia syndrome in pigs *Neth J Vet Sci* 1969 **2** 155-160
4 Berman M C, Harrison G G, Bull A B, Kench J E Changes underlying halothane induced malignant hyperpyrexia in Landrace pigs *Nature* 1970 **225** 653-655
5 Lucke J N, Hall G M, Lister D Porcine malignant hyperthermia I Metabolic and physiological changes *Br J Anaesth* 1976 **48** 297-304
6. Britt B A Malignant hyperthermia, a pharmacogenetic disease of skeletal and cardiac muscle *N Engl J Med* 1974 **290** 1140-1142
7 Harrison G G, Saunders S J, Biebuyck J F, et al Anaesthetic-induced malignant hyperpyrexia and a method for its prediction *Br J Anaesth* 1969 **41** 844-855
8 Gronert G A Malignant hyperthermia *Anesthesiology* 1980 **53** 395-423
9 Britt B A, Endrenyi L, Cadman D L, Ho M F, Fung H Y K Porcine malignant hyperthermia, effect of halothane on mitochondrial respiration and calcium accumulation *Anesthesiology* 1975 **42** 292-300
10 Moulds R F W, Denborough M A Biochemical basis of malignant hyperpyrexia *Br Med J* 1974 **2** 241-244
11 Britt B A Etiology and pathophysiology of malignant hyperthermia *Fed Proc* 1979 **38** 44-48
12 Somers C J, McLoughlin J V Malignant hyperthermia in pigs calcium ion uptake by mitochondria from skeletal muscle of susceptible animals given neuroleptic drugs and halothane *J Comp Pathol* 1982 **92** 191-198
13 Steward D J, Thomas T A Intracellular calcium metabolism and malignant hyperpyrexia In R A Gordon, B A Britt, W Kalow eds International Symposium on malignant hyperthermia. Springfield, Ill C C Thomas, 1973 409-414
14 Stadhouders A M Cellular calcium homeostasis, mitochondria and muscle cell disease In H F M Busch, J G I Jennekens, H R Scholte, eds Mitochondria and muscular diseases The Netherlands Mefar b v Beesterzwaag, 1981 77-88
15 Gallant E M, Godt R E, Gronert G A Role of plasma membrane defect of skeletal muscle in malignant hyperthermia *Muscle Nerve* 1979 **2** 491-494
16 Okumura F, Crocker B D, Denborough M A. Site of the muscle cell abnormality in swine susceptible to malignant hyperpyrexia *Br J Anaesth* 1980 **52** 377-383
17 Tandler C J, Libanati C S, Sandris C A The intracellular localization of inorganic cations with potassium pyroantimonate *J Cell Biol* 1970 **45** 355-366
18 Slocum R D, Roux S J An improved method for the subcellular localization of calcium using a modification of the antimonate precipitation technique *J Histochem Cytochem* 1982 **30** 617-629
19 Eikelenboom G, Minkema D Prediction of pale, soft exudative muscle with a non-lethal test for the halothane-induced porcine

malignant hyperthermia syndrome *Neth J Vet Sci* 1974 **99** 421-426
20 Verburg M P, Oerlemans F T J J, Bennekom C A, Gielen M J M, de Bruyn C H M M, Crul J F *In vivo* induced malignant hyperthermia in pigs I Physiological and biochemical changes and the influence of dantrolene sodium *Acta Anesthesiol Scand* 1984 **28** 1-8
21 Oberc M A, Engel W K Ultrastructural localization of calcium in normal and abnormal skeletal muscle *Lab Invest* 1977 **36** 566-577
22 Schäfer H J Cellular calcium and cell function Cytochemical, ultrastructural and x-ray microanalytical studies Stuttgart, New York Gustav Fischer Verlag, 1979
23 Barbi N C Detectability in energy dispersive microanalysis Scanning Electron Microscopy 1980/II SEM Inc A M F O'Hare (Chicago) IL 60666 USA 297-308
24 Dutson T R, Merkel R A, Pearson A M, Gann G L Structural characteristics of porcine skeletal muscle giant myofibers as observed by light and electron microscopy *J Anim Sci* 1978 **46** 1212-1220
25 Komnick H Elektronenmikroskopische Lokalisation von Na^+ und Cl^- in Zellen und Geweben *Protoplasma* 1962 **55** 414-418
26 Simson J A V, Spicer S S Selective subcellular localization of cations with variants of the potassium (pyro)-antimonate technique *J Histochem Cytochem* 1975 **23** 575-598
27 Klein R L, Yen S S, Thureson-Klein A Critique on the K-pyroantimonate method for semi-quantitative estimation of cations in conjunction with electron microscopy *J Histochem Cytochem* 1972 **20** 65-78
28 Clark M A, Ackerman G A A histochemical evaluation of the pyroantimonate-osmium reaction *J Histochem Cytochem* 1971 **19** 727-737
29 Yarom R, Chandler J A Electron-probe micro analysis of skeletal muscle *J Histochem Cytochem* 1974 **22** 147-154
30 Sugi H, Susuki S, Daimon T Intracellular calcium translocation during contraction in vertebrate and invertebrate smooth muscles as studied by the pyroantimonate method *Can J Physiol Pharmacol* 1982 **60** 576-587
31 Reed S J B Electron microprobe analysis London, New York Cambridge University Press, 1975
32 Wick S M, Hepler P K Selective localization of intracellular Ca^{2+} with potassium antimonate *J Histochem Cytochem* 1982 **30** 1190-1204
33 Bygrave F L Mitochondria and the control of intracellular calcium *Biol Rev* 1978 **53** 43-79
34 Carafoli E, Crompton M Regulation of intracellular calcium *Curr Top Membr Transp* 1978 **10** 151-216
35 Åkerman K E O, Nicholls D G Physiological and bioenergetic aspects of mitochondrial calcium transport *Rev Physiol Biochem Pharmacol* 1983 **95** 149-201
36 Crompton M, Capano M, Carafoli E The sodium-induced efflux of calcium from heart mitochondria A possible mechanism for the regulation of mitochondrial calcium *Eur J Biochem* 1976 **69** 453-462
37 Nicolls D Some recent advances in mitochondrial calcium transport *Trends Biochem Sci* 1981 **6** 36-38
38 Denton R M, Mc Cormack J G, Edgell N G Role of calcium ions in the regulation of intramitochondrial metabolism (Effects of Na^+, Mg^{2+} and ruthenium red on the Ca^{2+}-stimulated oxidation of oxoglutarate and on pyruvate dehydrogenase activity in intact rat heart mitochondria) *Biochem J* 1980 **190** 107-117
39 Lehninger A L Mitochondria and calcium ion transport *Biochem J* 1970 **119** 129-138
40 Ruitenbeek W, Verburg M P, Janssen A J M, Stadhouders A M, Sengers R C A *In vivo* induced malignant hyperthermia in pigs II Metabolism of skeletal muscle mitochondria *Acta Anaesthesiol Scand* 1984 **28** 14-26
41 Happel R D, Simson J A V Distribution of mitochondrial calcium *J Histochem Cytochem* 1982 **30** 305-311
42 Cheah K S, Cheah A M Mitochondrial calcium, erythrocyte fragility and porcine malignant hyperthermia *FEBS Lett* 1979 **107** 265-268
43 Cheah K S, Cheah A M Mitochondrial calcium transport and calcium-activated phospholipase in porcine malignant hyperthermia *Biochim Biophys Acta* 1981 **634** 70-84
44 Cheah K S, Cheah A M Skeletal muscle mitochondrial phospholipase A_2 and the interaction of mitochondria and sarcoplasmic reticulum in porcine malignant hyperthermia *Biochim Biophys Acta* 1981 **638** 40-49
45 Cheah A M Effects of long chain unsaturated fatty acids on the calcium transport of sarcoplasmic reticulum *Biochim Biophys Acta* 1981 **648** 113-119
46 Waite M, Scherphof G L, Boshouwers F M G, van Deenen L L M Differentiation of phospholipases A in mitochondria and lysosomes of rat liver *J Lipid Res* 1969 **10** 411-420
47 Nelson Th E, Schochet S S Malignant hyperthermia a disease of specific myofiber type, *Can Anaesth Soc J* 1982 **29** 163-167
48 Brownell A K W, Szabo M The *in vitro* caffeine contracture test influence of the muscle histochemical profile on test results *Can Anaesth Soc J* 1982 **29** 218-221
49 McLoughlin J V, Somers C J, Ahern A P, Wilson P The influence of neuroleptic drugs on the development of muscular rigidity in halothane sensitive pigs In Lunt G G, Marchbanks P M, eds The biochemistry of myasthenia gravis and muscular dystrophy London Academic Press, 1978 361-365

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CHAPTER 8

Malignant hyperthermia: Adenine incorporation and adenine metabolism in human platelets, influenced by halothane.

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MALIGNANT HYPERTHERMIA: ADENINE INCORPORATION AND ADENINE METABOLISM IN HUMAN PLATELETS, INFLUENCED BY HALOTHANE

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INTRODUCTION

Malignant hyperthermia (MH) is a pharmacogenetic disorder, elicited during general anaesthesia and carries a high mortality (1). MH is elicited in susceptible subjects by volatile anaesthetics like halothane, ether, enflurane and depolarizing muscle relaxants like succinylcholine(1). The inheritance of MH is autosomal dominant with incomplete penetrance and variable expression(1).

The screening for MH susceptibility is done by a pharmacological evaluation of a muscle specimen obtained by an open biopsy(1). The drawback of this test is its invasiveness and time consuming procedure. Moreover, due to its invasiveness it is an unsuitable test to be performed on children.

The need for a far less invasive test is obvious. A platelet bio-assay(2) has been advocated in a preliminary report as being useful identifying MH susceptible humans.

This report is stating the reduction of the incorporated adenine into nucleotides by the influence of halothane. Compared to the controls, a significant reduction is obtained in the ratio incorporated (ATP+ADP)/AMP(2).

Furthermore in a second preliminary report(3) it is stated that exposure to halothane produces a significant reduction of the platelet pool ATP and a significant increase in the hypoxanthine pool. These changes are expressed in the ratio: (ATP+ADP)/(AMP+Hypoxanthine). This

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ratio should show a significant drop in the halothane treated platelets compared to the untreated platelets of MH susceptible humans.

MATERIALS AND METHODS

The examined control subjects have no family history of MH. The 2 proven MH cases have been reported in the literature (4,5). We examined the parents of both patients and the parents of a 3rd case who died due to MH, according to the coroner's report.

Platelet rich plasma was prepared from venous blood collected in an ammonium heparinized vacuum tube, Vacuplast^{R}, (Greiner, Nürtingen, FRG). After centrifugation for 15 minutes at room temperature, 80 g, platelet rich plasma (PRP) was pipetted off with a plastic pipette into a polypropylene tube. Platelet counts were performed with a Coulter counter. Contamination of PRP was checked by means of a Haemalog-D. It was found to be less than 0.02%.

Incubation of PRP with ^{14}C-adenine was done in 1.6 ml Eppendorf tubes; 500 µl PRP, 20 µl ^{14}C-adenine (1µCi) (Radio Chemical Centre, Amersham, UK) spec.act.296 mCi/mmole. Increasing amounts of halothane (ICI, Macclesfield, UK) were added to the incubation mixture, leading to increasing concentrations of halothane from 0% to 2% (v/v).The concentration of adenine during the experiment was 6.8µM; incubation was performed at 37^{o}C for 20 min. in a shaking waterbath. After incubation a pellet was formed by centrifugation for 1 min. at 9,000 g at room temperature. The pellets were extracted with 40 µl trichloracetic acid (TCA) 6.5% at 0^{o}C for 10 min. After 1 minute of centrifugation at 9,000 g the TCA extract was used for thin layer chromatographic separation.

Thin layer chromatography (TLC) was performed on PEI-cellulose sheets (Macherey-Nagel, Düren, FRG). Two ascending development runs were needed with respectively 50% methanol for 40 minutes and, after drying, 0.4 M formic acid +0.25M LiCl for 25 minutes.

The spots were identified by means of UV light (254 nm), cut out and counted in a liquid scintillation counter (Packard 2450, USA).

High Performance liquid chromatography (HPLC). 500 µl PRP with increasing concentrations of halothane (0-2%(v/v)) were incubated in 1.6 ml Eppendorf tubes for 20 min. in a 37^{o}C shaking waterbath. After incubation the tubes were centrifuged for 1 min. at 9,000 g at room temperature and the pellet was extracted with 100 µl of

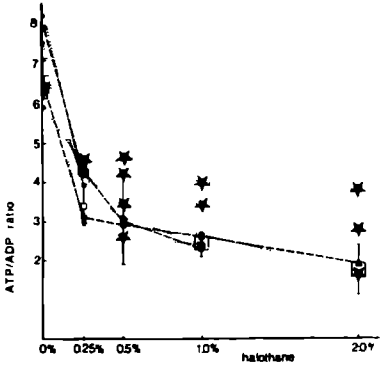

Figure 1
Incubation with ^{14}C-adenine
□= controls (n=4) bar:±sd.
●= proven MH patients (n=2)
✱= parents (n=5)

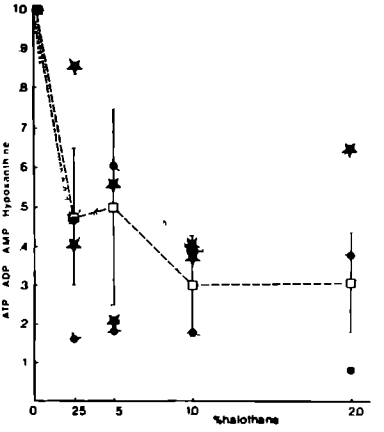

Figure 2
HPLC analysis.
□ = controls (n=12)
● = MH patients (n=2)
✱ = parents (n=2)

0.4 M perchloric acid. The extract was frozen and analyzed on the same day by means of HPLC. A Spectra Physics model SP 8000B (Spectra Physics, Sta Clara, Ca.,USA) equipped with a Bondapack-C18 column was used. Elution was performed with 0.1 M KH_2PO_4 at a constant flow rate of 1.5 ml/min. Peaks were monitored with a fixed wavelength UV detector (Spectra Physics) at 254 nm.

RESULTS AND DISCUSSION

The incubation experiments show a wide range of incorporation: 332-696 cpm adenine/10^6 platelets/20 min. Increasing amounts of halothane exert a dose related inhibition of adenine incorporation, both in the controls as well as in the patients and their parents. The values of incorporated adenine at 0% halothane in the patients range from 280-464 and in the parents from 136-784 cpm cpm adenine/10^6 platelets/20 min.

No discrimination can be made on the amount of incorporated adenine at 0% halothane from the patients, parents and controls.

The distribution of incorporated adenine in ATP and ADP is shown in fig.1. The ratio ATP/ADP at increasing concentrations halothane is expressed as a fraction of the ratio at 0% halothane.

No discrimination can be made on the ratio ATP/ADP from the controls, patients and their parents.

The results of the HPLC analysis of adenine nucleotides of platelets incubated with increasing concentrations of halothane is shown in fig.2. The ratio (ATP+ADP)/(AMP+Hypoxanthine) at increasing concentrations of halothane is expressed as a fraction of the ratio at 0% halothane.

No discrimination can be made between the controls, patients and their parents from the drop in the ratio (ATP+ADP)/(AMP+Hypoxanthine)on exposure to halothane.

CONCLUSION

Both the ^{14}C-adenine incorporation as well as the HPLC analysis of adenine nucleotides in platelets exposed to increasing concentrations of halothane, are unable to provide a significant discriminating effect, separating the MH susceptible subjects from the controls.

REFERENCES

1. G.A. Gronert, Malignant hyperthermia, Anesthesiol., 53:395.
2. C.C. Solomons, S. Tan and J.A. Aldrete, Platelet metabolism and malignant hyperthermia, in:"Malignant Hyperthermia, 2nd International Symposium", J.A. Aldrete and B.A. Britt, eds.; Grune and Stratton, New York (1978) p. 221.
3. C.C. Solomons, N. McDermott and M. Mahowald, Screening for malignant hyperthermia with a platelet bioassay, N. Eng. J. Med. 303:642 (letter to the editor) (1980).
4. J. Van Der Nat, Hyperpyrexia tijdens narcose (1968), Ned. T.v.Gen. 112, 2227 (1968).
5. F. Hoevenaars, R.C.A. Sengers, A.M. Stadhouders, M.J.M. Gielen, W.L.C. Brunings, J.M.F. Trijbels and H. Bookelman, Maligne hyperpyrexia: een vaak niet herkende complicatie van narcose. Ned.T.van Gen. 121, 976, (1977).

CHAPTER 9: METABOLITES IN SKELETAL MUSCLE BIOPSIES FROM HUMANS SUSCEPTIBLE TO MALIGNANT HYPERTHERMIA AND CONTROLS

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Introduction

Malignant hyperthermia (MH) is a pharmacogenetic disease triggered by volatile anaesthetics and/or depolarising skeletal muscle relaxants[1]. Mortality is still about 30%, inspite of the availability of a specific curative drug, dantrolene sodium[2]. The incidence of an MH crisis is estimated to range from 1/15,000 delivered anaesthesias in children, 1/50,000 to 1/100,000 in adults[3-4]. The inheritance is thought to be autosomal dominant, with variable expression and reduced penetrance . However, no uniform opinion exists on its inheritance pattern, partly due to the limitations of the available screening method for a mass screening procedure. At present the screening method of choice is a pharmacological in-vitro test of a skeletal muscle, obtained by an open biopsy[3]. On exposure to successive increasing concentrations of caffeine or halothane, a contracture is observed in skeletal muscle

obtained from MH susceptible subjects at lower concentrations compared to controls[1,5,6]. However, the major drawback of the test is its invasiveness. The surgical intervention to obtain a muscle specimen as well as the time consuming procedure in the laboratory to perform the in-vitro test, makes this test unsuitable for mass screening.

A possible alternative might be the analysis of skeletal muscle metabolites. Studying on the porcine MH model, it was found that MH susceptible pigs showed a different skeletal muscle metabolite profile compared to controls[7,8]. In muscle obtained from MH susceptible pigs a decreased concentration was measured in phosphocreatine (PCr) and an increased concentration of lactate and creatine (Cr)[8]. Furthermore, other workers have shown increased concentrations of glycolytic intermediates in muscle from MH susceptible individuals[9].

These findings stimulated us to evaluate a number of skeletal muscle metabolites in humans as possible diagnostic parameters for MH.

Materials and methods

All skeletal muscle biopsies were taken at the Toronto University Hospital from individuals requesting for MH screening. The standardized procedure included the use of diazepam, fentanyl for induction endotracheal intubation and ventilation with oxygen (F_iO_2 : 0.3), and nitrous oxyde, delivered from a vapour free anaesthesia machine.

The muscle biopsies were taken from the m.vastus lateralis and immediately frozen and panacked by a pre-cooled brass clamp at -70 °C. The sample was stored at -40 °C in a refrigerator. Additional muscle samples were taken to perform the routine in-vitro caffeine contracture test, in order to establish MH susceptibility[5,6]. This test was performed according to the description of Kalow and Britt[6,10].
The criteria to define MH susceptible subjects and controls were according to the protocol employed at the Dept. of Anaesthesia, Toronto, Canada[6]. The basic criterium hereby is the caffeine specific concentration (CSC): the dose of caffeine in mM required to raise the resting tension of a muscle fascicle by 1 gram. The CSC for non MH susceptible subjects is 4.1 mM of caffeine or greater, and in combination with halothane 1%: 1.2 mM caffeine or greater.
An intermediate group has been identified, showing a normal response to caffeine but an MH response to halothane 1% plus caffeine. The latter group is called the K-type group[11].

Analysis of skeletal muscle metabolites was performed on the freeze-clamped skeletal muscle, shipped on dry ice from Toronto to Nijmegen, The Netherlands.
Before shipping the samples were coded, so the analysis was performed without knowing which sample was from an MH susceptible subject or from a control.
The skeletal muscle metabolites were studied using an isotachophoric technique[7,12]. A muscle sample was freeze

dried and homogenized mechanically. Extraction of muscle metabolites was performed using ice-cold EDTA 1,25 mM (pH 7.6) in 50% methanol at 0 °C, for 15 minutes. The suspension was centrifuged for 1 minute, at 10,000 g, and the supernatant freeze-dried. To this lyophilized fraction, 200-800 µl of distilled water was added. Analysis was performed by injection of 5 µl of this extract into a Tachophor^{R}, LKB Instruments, Bromma, Sweden. Temperature during the measurement was kept at 20 °C, and u.v. absorbtion was measured at 254 nM. The concentrations measured in the extracts were expressed on the basis of the wet weight of the muscle sample[8].

Creatine was measured in a neutralized perchloric acid extract of a muscle specimen, applying the enzymatical system described by Jaworek et al, with the addition of creatine kinase and adenosine triphosphate (ATP)[13].

After all analyses had been performed, the code was broken and the data were submitted to statistical evaluation, using the analysis of variance, and Student's T-test.

Results

Based on the criteria mentioned in "materials and methods" a division was made into three groups according to the reaction on caffeine alone and the combination halothane 1% plus caffeine. This division is displayed in table 1, as well as the skeletal muscle metabolite values belonging to each group. No significant differences were observed

with respect to the parameters shown, comparing the control with the MH susceptible subjects (MHS) or the K-type reactors. Especially PCr, Cr and lactate values were not significantly different in controls and MH susceptible subjects.

Table 1 :

Skeletal muscle metabolites in malignant hyperthermia susceptible (MHS) humans, non MH susceptible (controls) humans and those belonging to an inter-mediate group (K-type). All values are expressed in nmol/mg wet weight, mean value + SD

Parameter	P-value	Controls (N=31)	K-type (N=18)	MHS (n=19)
ATP	.44	3.75 ± 0.74	3.89 ± 0.43	3.98 ± 0.61
ADP	.28	0.14 ± 0.09	0.13 ± 0.08	0.11 ± 0.05
AMP	.18	0.05 ± 0.04	0.04 ± 0.04	0.03 ± 0.02
IMP	.18	0.14 ± 0.13	0.09 ± 0.07	0.09 ± 0.07
NAD^+	.81	0.13 ± 0.10	0.11 ± 0.10	0.12 ± 0.09
PCr	.51	13.20 ± 3.33	14.42 ± 3.85	13.74 ± 3.62
Cr	.43	16.03 ± 4.93	15.06 ± 3.88	16.94 ± 3.84
Cr/(Cr + PCr)	.17	0.54 ± 0.06	0.51 ± 0.06	0.55 ± 0.09
Lactate	.97	3.73 ± 1.67	3.79 ± 1.92	3.65 ± 1.74
PCr/lactate	.91	4.41 ± 2.36	4.68 ± 2.28	4.61 ± 2.43
Pi	.66	7.42 ± 1.92	7.24 ± 1.40	6.97 ± 1.61

ATP, ADP and AMP: adenosine trihphosphate, -di phosphate and -mone phosphate, IMP: inosine monophosphate, NAD^+: nicotinamide adenine dinucleotide oxidized, PCr: phosphocreatine, Cr: creatine, Pi: inorganic phosphate.

Statistical evaluation by means of analysis of variance (P-value) did not show significant differences in metabolites levels between

the three groups.

Discussion

The analysis of skeletal muscle metabolites can be useful for the diagnostic evaluation of inherited skeletal muscle diseases, like Duchenne muscular dystrophy[12]. A major advantage of the isotachophoretic analysis of skeletal muscle metabolites is the small amount of biopsy material needed. A muscle sample of 10-25 μg provides satisfactory and reproducible separations of a considerable number of essential metabolites within the same run[12]. Current enzymatic methods need bigger muscle samples and the measurement of most metabolites is indirect and more laborious.

Our previous studies on the porcine MH model showed that on the basis of skeletal muscle metabolite analysis a differentiation could be made between MH susceptible and control pigs[8]. Furthermore, studies by others on humans susceptible to MH have provided some indications of a pre-existent difference in skeletal muscle metabolites, compared to controls[9]. In the latter study significantly lower concentrations were measured in MH susceptible muscle of ATP, PCr and an increased concentration of glucose-6-phosphate. These results and our previously obtained results on the porcine MH model have stimulated us to investigate skeletal muscle metabolites in humans at risk for MH, in order to determine whether abnormal levels of metabolites might be encountered.

The results obtained of skeletal muscle metabolites values are well comparable to values reported by others on muscle biopsies taken by a biopsy needle[14]. The latter reported values for ATP of 3.92 ± 0.34 nmol/mg wet weight, for PCr 10.17 ± 1.09 nmol/mg wet weight and lactate 3.33 ± 0.41 nmol/mg wet weight. However, a major criticism on the analysis of skeletal muscle metabolite analysis by means of needle biopsy, is the too long time needed for freezing the sample.

It has been stated that freezing of skeletal muscle samples has to occur within 100 msec to avoid breakdown of PCr during the freezing process[15]. The best possible way to do so is to put a liquid nitrogen cryoprobe into the muscle mass. However, for ethical reasons this is not feasable, as the mass to be frozen will be much bigger than needed for a needle biopsy sample. Furthermore, chemical irritation may occur due to the liquid nitrogen. In addition, changes in skeletal muscle metabolite levels can be detected by using the conventional needle biopsy sampling[16]. Therefore we think it is allowed to present our data on skeletal muscle metabolites and to draw conclusions from the results obtained.

In contrast to the findings in our pig study no significant differences were measured in skeletal muscle metabolites analysed. The PCr value is the first one to present a decreased level on an increased energy demand, or a decreased production capacity. In the MH samples this is

not the case. This supports the hypothesis that MH is not a pre-existent disease but rather an induced metabolic disorder. This statement has recently been supported by using a nuclear magnetic resonance (NMR) technique, measuring PCr and ATP changes in pigs prior to and during an MH crisis [17]. On induction of the MH crisis with succinylcholine, a drop occurred in PCr levels, whereas prior to the administration of succinylcholine no significant differences in PCr levels were measured.

Literature :

1. Gronert G A. Malignant hyperthermia. Anesthesiology 1980: 53, 395-423.
2. Britt B A. In: Malignant hyperthermia, preface. Britt B A ed. Int Anesthes Clin 1979: 17, vii-x.
3. Nelson T E, Flewellen E H. Current concepts, the malignant hyperthermia syndrome. N Engl J Med 1983: 309, 416-418.
4. Britt B A, Kalow W. Malignant hyperthermia: a statistical review. Can Anaesth Soc J 1970: 17, 293-315.
5. Kalow W, Britt B A, Terreau M E, Haist C. Metabolic error of muscle metabolism after recovery from malignant hyperthermia. Lancet 1970: ii, 895-898.
6. Kalow W, Britt B A, Richter A. The caffeine test of isolated human muscle in relation to malignant hyperthermia. Can Anaesth Soc J 1977: 24, 678-694.
7. Mitchell G, Heffron J J A, Van Rensburg A A J. A halothane induced biochemical defect in muscle of normal and malignant hyperthermia susceptible landrace pigs. Anesth Analg 1980: 59, 250-256.
8. Verburg M P, Oerlemans F T J J, Van Bennekom C A, Gielen M J M, De Bruyn C H M M, Crul J F. In vivo induced malignant hyperthermia in pigs. I, physiological and biochemical changes and the influence of dantrolene sodium. Acta Anaest Scand 1984: 28, 1-8.
9. Isaacs H, Heffron J J A. Morphological and biochemical defects in muscle of human carriers of the malignant hyperthermia syndrome. Br J Anaesth 1975: 47, 475-481.

10. Britt B A, Endrenyi L, Kalow W, Peters L. The adenosine triphophate (ATP) depletion test: comparison with the caffeine contracture test as a method of diagnosing malignant hyperthermia susceptibility. Can Anaesth Soc J 1976: 23, 624-635.
11. Nelson T E, Flewellen F H. Spectrum of susceptibility to malignant hyperthermia. Anesth Analg 1983: 62, 545-552.
12. Oerlemans F T J J, Van Bennekom C A, De Bruyn C H M M. Metabolite profiling of human muscle extracts by isotachophoresis. J Inher Metab Dis 1981: 4, 109-110.
13. Jaworek D, Gruger W, Bergmeyer H U. In: Methoden der Enzymatische Analyse II, Bergmeyer ed. Weinheim: Verlag Chemie, 1974: 2178-2181.
14. Essen B, Hagenfeldt L, Kayser L. Utilization of blood-borne intramuscular substrates during continous and intermittent exercise in man. J Physiol 1975: 251, 303-315.
15. Kretzschmar K M, Wilkie D R. A new approach for freezing tissues rapidly. J Physiol 1975: 251, 303-315.
16. Edwards R H T, Dawson M J, Wilkie D R, Gordon R E, Shaw D. Clinical use of nuclear magnetic resonance in the investigation of myopathy. Lancet 1982: i, 725-731.
17. Roberts J T, Burt T, Gouylai L, Chance B, Screter F, Ryan J. Immediate uncoupling of high energy phosphorylation in muscle of malignant hyperthermic swine determined non-invasively by whole body 31P nuclear magnetic resonance. Anesthesiology 1983: 59, A 230.

CHAPTER 10: THE USE OF A REGIONAL ANAESTHETIC TECHNIQUE, THREE-IN-ONE BLOCK, TO TAKE MUSCLE BIOPSIES IN HUMANS FOR THE EVALUATION OF MALIGNANT HYPERTHERMIA SUSCEPTIBILITY

The in-vitro caffeine contracture test (CCT) is at present indispensible to confirm or exclude susceptibility to malignant hyperthermia (MH) in humans[1-6]. Screening for MH is indicated for those having a family history suggestive of an MH crisis. The CCT is a useful method in counselling members of an MH affected family. When the patient is aware of his susceptibility to MH, accidents can be avoided by informing the anaesthetist about the susceptibility to MH. In that case all precautions can be taken and the use of possible MH triggering agents will be avoided.

The basis of the CCT is the increased sensitivity of skeletal muscle obtained from MH susceptible subjects to caffeine and halothane[1-6]. On exposure to caffeine or halothane, muscle samples obtained from MH susceptible humans produce a contracture at lower concentrations compared to muscle samples obtained from controls. Muscle samples from MH susceptible subjects produce a contracture at caffeine concentrations below or equal to 2 mM, and the control samples at 3 mM or higher. For halothane the values are 1% halothane or less for muscle specimens from MH susceptible subjects, and 3% or higher for controls. A contracture is a sustained contraction of skeletal muscle, without electrical activity

and no tendency to relax spontaneously. The lowest concentration of caffeine or halothane producing a sustained rise of the base line tension of at least 0.2 gram, produced by a muscle specimen weighing about 50-100 mg, and is called threshold concentration.

Besides the confirmation or exclusion of MH susceptibility, the study reported here was designed to evaluate the effectiveness of a regional anaesthetic technique in taking a skeletal muscle biopsy from the m.vastus medialis.

Material and methods

Patient selection was limited to those people asking for a counselling concerning MH susceptibility, based on a family history of probable MH (n=21). The history of the MH crisis was re-examined, as far as possible using medical records. Special attention was paid to possible transfusion reaction or septicaemia.

Creatine phosphokinase (CK) was measured in venous blood samples using standard laboratory techniques. CK measurement was done when the subject had restrained from heavy physical exercise for at least three days. Thereafter skeletal muscle biopsies were taken from the m.vastus medialis using a regional anaesthetic technique. This was performed as an out-patient procedure. After an oral premedication with benzodiazepam 10 mg, a regional anaesthesia was performed in a leg, using the "three-in-one" block as described by Winnie et al[7].

The local anaesthetic used was prilocaine: 40 ml of a 1.5% solution with epinephrine added, 1/200.000. After a 20-30 minutes observation period, satisfactory analgesia was obtained, measured by skin-prick using a hypodermic needle. Monitoring of the patient consisted of taking ECG, periferal pulse and bloodpressure.

A biopsy was taken from the m.vastus medialis measuring 3 x 0.5 x 0.5 cm. The biopsy was fixed in-situ in a specially constructed clamp, composed of two artery forceps, welded together at a fixed distance of 3 cm.. This was done to avoid retraction of the muscle specimen after removal from the skeletal muscle mass. The sample was taken parallel to the longitudinal axis of the muscle fibres, in order to obtain a maximal number of viable muscle fibres. Additional muscle tissue was taken for routine histological examination and muscle fibre typing.

The muscle sample for the in-vitro CCT was immediately transferred to the laboratory in a Krebs-Ringer solution. In the laboratory intact muscle fibres measuring 20 x 2 x 2 mm were prepared from the biopsy specimen. This muscle fascicle was fixed on one end and the other end attached to a force displacement transducer, Gould-UC-3. The force obtained was registered with a Grass polygraph. The muscle fascicle was directly stimulated supramaximally, 1 ms, 0.1 Hz, to asses viability. After obtaining a stable base line and maximal twitch amplitude, the experiment was continued. During the whole procedure the tissue bath was kept at 37 ^{o}C, aerated

with carbogen (95% O_2 + 5% CO_2), pH 7.4.

The experiment consisted of two runs. The first one was a static run with caffeine, the second one was a dynamic run with halothane. The static caffeine run was performed with succesively increasing concentrations of caffeine: 0.5, 1.0, 2.0, 4.0, 8.0 and 16 mM. The muscle sample was exposed for at least 3 minutes to each caffeine concentration, before the next caffeine concentration was used. All caffeine concentrations were dissolved in a Krebs-Ringer solution. When a contracture developed (a substained) increase of tension), the next concentration of caffeine was administered as soon as the maximal tension was obtained. In this way a cumulative dose response reaction was obtained. The lowest concentration of caffeine producing a contracture was called the threshold concentration.

In case of no twitch response on direct electrical stimulation, the muscle sample was considered not viable and discarded.

The second run, the "dynamic halothane test" was performed by exposing the muscle sample to succesively increasing concentrations of halothane. On exposure to each concentration of halothane, the muscle sample was stretched over a fixed distance and after 3 minutes relaxed by returning to the initial length. At each succesive concentration of halothane the same distance was used. At first the muscle sample was exposed to 0% halothane, administered by passing the carbogen through a calibrated vaporizer. The

characteristic length-tension pattern of the last cycle at 0% halothane was used as reference value. On exposure to halothane the following characteristics were observed and considered diagnostic for MH susceptibility.

I . A contracture develops on exposure to halothane

II. The tension obtained after completion of the stretching procedure is greater than the tension observed at 0% halothane.

Results of the three-in-one block

The three-in-one block using prilocaine as a local anaesthetic agent, produced good analgesia of the skin overlying the biopsy spot in all patients. However, in two patients we had to give additional intravenous analgesics, due to pain on traction of the skeletal muscle. All 21 individuals examined did not show any adverse reactions to the surgery and anaesthesia.

Results of the in-vitro test: static caffeine test

A typical registration of the static caffeine test in a non-MH-susceptible subject is shown in figure 1-A. A contracture is observed on exposure to caffeine 4 mM and higher concentrations of caffeine. In figure 1-B a registration is presented of the reaction of a muscle specimen obtained from an MH susceptible individual. A contracture develops on the addition of caffeine 1 mM, and higher concentrations of caffeine.

FIGURE 1A :

Registrations are shown of the tension produced by a skeletal muscle fascicle obtained from the m.vastus medialis from a non-MH-susceptible human. The muscle fascicle is fixed on one end, the other end is attached to a force displacement transducer. The muscle fascicle is bathed in a Krebs-Ringer solution, 37 ^{o} C, pH 7.4 and continuously aerated with carbogen (95% O_2 + 5% CO_2). The muscle fascicle is directly stimulated, supramaximally 0.1 Hz, 2 m sec to asses viability. The muscle sample is exposed for 5 minutes to each successive increasing concentration of caffeine. On exposure to caffeine 4 mM a contracture develops: a sustained elevation of the baseline tension.

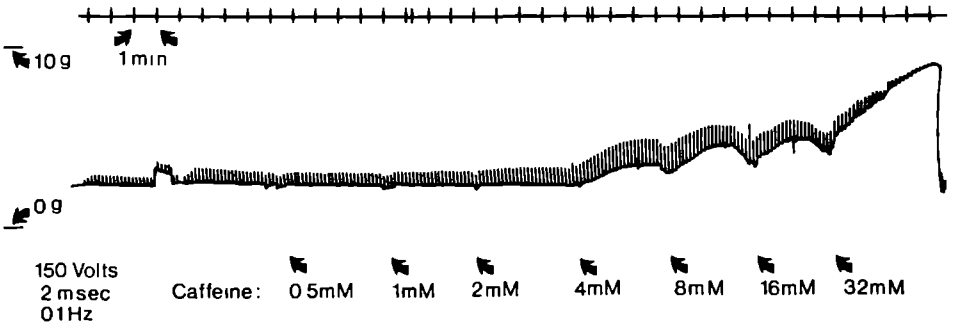


FIGURE 1B :

Identical bathing conditions as in figure 1A. It can be noted that on addtion of caffeine 1 mM a contracture develops. This is considered to be a response of a skeletal muscle obtained from a human susceptible to malignant hyperthermia.

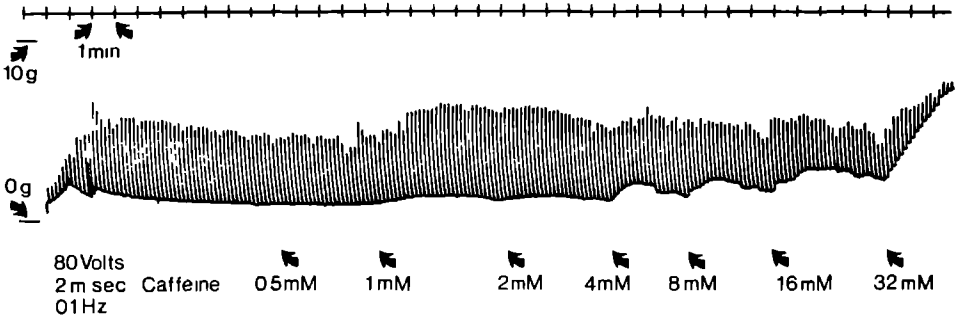
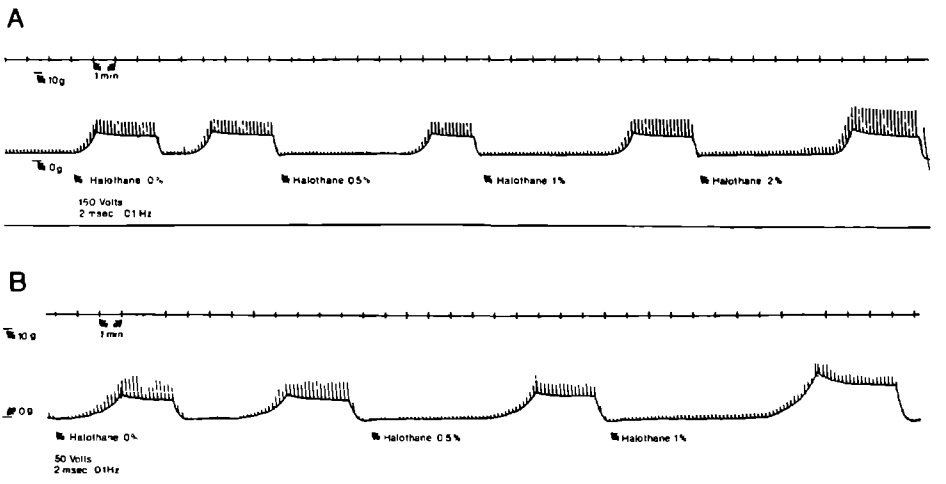


Figure 2A (top). Tension response as traced in the dynamic halothane test performed on a skeletal muscle sample obtained from a non-MH-susceptible human.

Figure 2B (bottom). Tension response as traced in the dynamic halothane test performed on a skeletal muscle sample obtained from an MH susceptible human.



Dynamic halothane test

A typical registration of the dynamic halothane test performed on a skeletal muscle sample obtained from a non-MH-susceptible subject is shown on figure 2-A. No contracture developed on exposure to halothane as high as 2%. No increase was measured of the tension at completion of the stretching procedure. In figure 2-B a registration is shown of the reaction of a muscle specimen obtained from an MH susceptible subject. On exposure to halothane 1% a contracture develops of the baseline tension. Furthermore, after completion of the stretching procedure a higher tension is obtained compared to the tension produced at 0% halothane. A summary of the in-vitro test is shown in table 1, presenting the caffeine and halothane threshold concentrations producing a contracture.

Histological examination

The histological examination of the subjects examined did not provide specific histological abnormalities in the MH susceptible subjects compared to the non-MH-susceptible subjects. A summary of the histological examiniations is shown in table 2, presenting the distribution of fibre types.

Creatine-phosphokinase analysis

Serum levels were elevated in 2 out of the 5 subjects considered MH susceptible. In the non-MH-susceptible group 3 out of 16 showed an increased CK value. One of them was shown to be affected by myotonia dystrophica. The CK values measured in the patients examined is shown in table 2.

Table 1 :

Results of the in-vitro contracture test. A static run is performed with caffeine, and a dynamic run is performed using halothane. The lowest concentration of caffeine and halothane producing a contracture of 0.2 g or more, is called the threshold concentration. All 21 subjects examined had a family history of possibly MH.

Individuals	Sex	Age	Caffeine	Halothane	Conclusion
B-C	F	36	4 mM	neg. at 2%	MH^-
S	M	32	4 mM	neg. at 2%	MH^-
vS-S	F	34	4 mM	neg. at 2%	MH^-
HS	F	28	4 mM	neg. at 2%	MH^-
vE	M	23	4 mM (myotonia dystrophica)	neg. at 2%	MH^-
S-O	F	34	4 mM	neg. at 2%	MH^-
K-O	F	28	4 mM	neg. at 2%	MH^-
M-P	F	46	4 mM	neg. at 2%	MH^-
D	M	24	4 mM	neg. at 2%	MH^-
vV-O	F	30	4 mM	neg. at 2%	MH^-
E	M	28	4 mM	neg. at 2%	MH^-
E-vL	F	40	4 mM	neg. at 2%	MH^-
G-vL	F	34	4 mM	neg. at 2%	MH^-
K-vL	F	43	4 mM	neg. at 2%	MH^-
HvL	M	36	4 mM	neg. at 2%	MH^-
E-vB	F	39	4 mM	neg. at 2%	MH^-
E-T	F	47	1.0 mM	pos. at 0.5%	MH^+
M-vL	F	27	0.5 mM	pos. at 0.5%	MH^+
M-vL	F	39	2.0 mM	pos. at 1.0%	MH^+
F-vL	M	41	1.0 mM	pos. at 0.5%	MH^+
B-vL	F	38	0.5 mM	pos. at 0.5%	MH^+

Malignant hyperthermia susceptible : MH^+

Non-malignant hyperthermia susceptible: MH^-

Table 2 :

Results of the histological and creatine phosphokinase (CK) analysis of the 21 subjects mentioned in table 1.
Normal CK value less than 90 I.U./l. Normal distribution of fibre type in human m.vastus medialis; type 1:35-60%, fibre type 2: 50-65%, intermediate till 5%, central nuclei till 3%.

Individual	Sex	Age	Histology			CK
			% type 1	% type 2	% intermediate	
B-C	F	36	46.7	51.7	1.7	40
S	M	32	34.7	64.2	1.0	208
			(central nuclei 6.2%)			
vS-S	F	34	64.7	35.3	-	59
HS	F	28	41.5	58.3	0.2	44
vE	M	23	40.0	60.0	-	391
			(myotonia dystrophica)			
S-O	F	34	58.6	41.4	-	38
K-O	F	28	42.3	57.7	-	57
M-P	F	46	-	-	-	57
D	M	24	47.2	48.4	4.4	-
vV-O	F	30	43.0	56.6	0.4	40
E	M	28	34.7	63.8	1.4	169
			(central nuclei 4.9%)			
F-vL	F	40	55.2	44.8	-	44
G-vL	F	34	63.7	35.6	0.7	37
			(central nuclei 8.9%)			
K-vL	F	43	35.5	64.5	-	34
H-vL	M	36	24.6	55.1	20.3	18
E-vB	F	39	39.4	60.0	0.6	79
			(central nuclei 8.2%)			
E-T	F	47	43.5	56.5	-	200
M-vL	F	27	36.7	59.8	3.5	105
M-vL	F	39	48.9	51.1	-	33
F-vL	M	41	28.4	71.6	-	52
B-vL	F	38	31.5	68.2	0.3	48

Discussion

At present agreement exists that the only reliable diagnostic test for susceptibility to malignant hyperthermia is the in-vitro contracture test [1,6]. Exposure to caffeine or halothane of a viable skeletal muscle sample enables us to establish MH susceptibility, based on the contracture inducing concentration of halothane or caffeine. In our study the non-MH-susceptible subjects were considered those, reacting at a caffeine concentration of 4 mM or higher as well as no contracture response at exposure to halothane 2%. The MH susceptible subjects were those showing a contracture at exposure to caffeine, below a concentration of 4 mM. Based on the caffeine exposure we have considered 5 subjects MH susceptible. Those 5 subjects also showed a contracture on exposure to halothane, 4 at 0.5% and all 5 at 1%. The dynamic halothane test extends the information about the reaction pattern to halothane. The increase of tension after completion of the stretching procedure is clearly visible and more marked than the halothane contracture at resting length.

The histological examiniation did not provide evidence of a specific histological abnormality in the MH susceptible subjects. The non-MH-susceptible subjects, 4 of them, showed an increased presence of central nuclei: 4.9%, 6.2%, 8.2% and 8.9% respectively, less than 3% is considered normal. Furthermore, one of the non-MH-susceptible subjects presented later on to be affected by myotonia dystrophica. This obser-

vation points to the specificity in this case of the in-vitro contracture test, as this subject affected by myotonia dystrophica had a normal response to caffeine and halothane.

In our study we have in the MH susceptible group one patient showing a too low percentage of fibre type 1. This subject, however, showed a caffeine threshold concentration of 1.0 mM caffeine and for halothane 0.5%. In-vitro studies on skeletal muscle obtained from MH susceptible pigs showed a dependency of the caffeine threshold concentration regarding the composition of fibre types[8]. Skeletal muscle predominantly composed of fibre type 2 (80%), showed a lower caffeine threshold compared to a muscle from the same animal, composed of about a 50/50 distribution of fibre type 1 and 2[8]. Comparable results have been obtained on exposure of skeletal muscle of rats. Muscles composed primarily of fibre type 1 developed contractures at lower concentrations of caffeine compared to muscle composed primarily of fibre type 2[9].

The CK value did not provide specific relation to MH susceptibility. Of the 5 cases considered MH susceptible, only 2 had an elevated CK value. Furthermore, in the non-MH-susceptible group, 3 of them showed an elevated CK value, 1 of them was the subject suffering from myotonia dystrophica.

In our study we have used a regional anaesthetic technique

with an amide-type local anaesthetic. In the literature, the use of ester-type local anaesthetics is advocated, based on studies in-vitro on frog skeletal muscle preparations [10,11]. In these studies lidocaine 3.67 mM (858.8 mg/l) potentiated a caffeine induced contracture, whereas procaine 3.67 mM inhibited the caffeine induced contracture in frog muscle at pH 7.4, room temperature [10]. However, the potentiating effect of lidocaine on the caffeine induced contractures showed to be pH dependant. At pH 6.0 lidocaine 3.67 mM did not potentiate or antagonise the caffeine induced contractures. At pH 7.2 a potentiating of the contracture was seen and at pH 8.2 lidocaine 1 mM was as effective as lidocaine 3.67 mM to potentiate the caffeine induced contracture [10]. The concentrations lidocaine used are far beyond the toxic range in humans: 5-6 mg/l. So the recommendation not to use amide-type local anaesthetics is questionable.

Furthermore, infusion of amide-type local anaesthetics in MH susceptible pigs failed to produce an MH crisis[12]. On intravenous administration of lidocaine as a bolus injection of 2-3 mg/kg body weight, followed by an infusion rate of 120 ug/kg body weight/min for 10 minutes, no signs of a MH crisis were observed. The peak plasma concentrations of lidocaine were 7 mg/l [12].

The infusion of bupivacaine intravenously at a rate of 1 mg/kg body weight in MH susceptible pigs, did not produce an MH reaction either[12]. The peak-plasma concen-

trations ranged from 3-4 mg/l, well exceeding the toxic concentration in humans: 1.6 mg/l [13].

Based on these observations of the infusion of amide-type local anaesthetics, without producing MH in pigs and the vulnerability of the recommendation of the use of ester-type local anaesthetics based on in-vitro frog skeletal muscle preparations, we thought it justified to use an amide-type local anaesthetic. Furthermore, the use of a regional anaesthetic procedure is advisable in MH susceptible humans to avoid stress induced by fear for a general anaesthesia. We have choosen to use the amide-type local anaesthetic, prilocaine, as it is preferentially used for performing a regional block into vascular areas. Furthermore, the local neural tissue toxicity is less than lidocaine. Using 600 mg with epinephrine added, no untoward reactions were observed, especially not in the 5 patients considered MH susceptible.

In conclusion we may say that the in-vitro contracture test with halothane and caffeine is a useful method to diagnose MH susceptibility. The CK value and histological examination does not provide specific abnormalities, regarding MH susceptibility. The use of amide-type local anaesthetics in MH susceptible humans should not be discouraged.

Literature :

1. Gronert G A. Malignant hyperthermia. Anaesthesiology 1980: 53, 395-423.
2. Rosenberg H, Reed S. In-vitro contracture tests for susceptibility to malignant hyperthermia. Anesth Analg 1983: 62, 415-420.
3. Nelson T E, Flewellen F H, Gloyna D F. Spectrum of susceptibility to malignant hyperthermia-diagnostic dilemma. Anesth Analg 1983: 62, 545-552.
4. Britt B A. Preanesthetic diagnosis of malignant hyperthermia, International Anesthesiology Clinics. Boston, Little & Brown Co, 1979: 17, 63-96.
5. Ellis R F, Harriman D G F, Currie S, Pain P A. Screening for malignant hyperthermia in susceptible patients. In: Aldrete J A, Britt B A, eds. Second International Symposium on malignant hyperthermia. New York: Grune & Stratton 1978: 273-285.
6. Nelson T E, Flewellen F H. Current concepts: the malignant hyperthermia syndrome. New Eng J Med 1983: 309, 416-418.
7. Winnie A P, Ramamurthy S, Durrani Z. The inguinal paravascular technic of lumbar lexus anesthesia: the 3-in-1 block. Anesth Analg 1973: 52, 989-994.
8. Nelson T E, Schochet S S. Malignant hyperthermia: a disease of specific myofibre type? Can Anaesth Soc J 1982: 29, 163-167.
9. Brownell A K W, Szabo M. The in-vitro caffeine contracture test: influence of the skeletal muscle histochemical

profile on the test results. Can Anaesth Soc J 182: 29, 218-221.

10. Feinstein M B. Inhibition of caffeine rigor and radio-calcium movements by local anesthetics in frog sartorius muscle. J Gen Physiol 1963: 47, 151-172.
11. Strobel G E, Bianchi C P. An in-vitro model of anaesthetic hypertonic hyperpyrexia, halothane-caffeine-induced contractures. Anesthesiology 1971: 35, 465-473.
12. Harrison G G, Morell D F. Response of MHS swine to i.v. infusion of lignocaine and bupivacaine. Br J Anaesth 1980: 52, 385-387.
13. Scott D B, Cousins M J. Clinical pharmacology of local anesthetic agents. In: Cousins M J, Bridenbaugh P O eds. Neural blockade in clinical anesthesia and management of pain. Philadelphia-Toronto J B Lippincott, 1980: 86-121.

Malignant hyperthermia (MH) is a life threatening complication of anaesthesia, based on a pharmacogenetic predisposition of the subject concerned. Factors contributing to the mortality are the rarety of the syndrome -- every anaesthetist may meet only 1 to 5 cases during his or her professional life -- and the late and non-specific signs and symptoms of an MH crisis[1,2]. Furthermore, the progress of the MH crisis is fulminant, leading to severe hypoxia and profound disturbance of the acid-base status and finally to irreversible changes leading to the death of the subject or severe neurological deficits. Based on our studies on the porcine MH model, we have concluded that continuous measurement of expiratory CO_2 in a patient receiving general anaesthesia, will give an early indication of the development of an MH crisis[3]. This increased expiratory CO_2 output can easily be measured by a capnometer. In our porcine MH studies, expiratory CO_2 was doubled within 5 minutes after the administration of halothane plus succinylcholine[3]. Compared to the temperature elevation, muscle temperature was elevated 0.6 ^{o}C and core temperature only 0.3 ^{o}C, 5 minutes after induction of the MH crisis. This increased CO_2 output may be considered as a specific sign of MH. In this way the suspicion of an impending MH crisis is risen very early. Appropriate treatment can be started in time.

Dantrolene sodium proved to be an effective drug to

reverse the MH crisis in our porcine MH study: all MH susceptible pigs survived to MH crisis. This was only possible due to the early start of the treatment, 10 minutes after the induction of the MH crisis. A dose of 1 mg/kg body weight proved to be effective. The time factor is an important one, as on another occasion, we started treatment of an MH crisis in a pig after about 20 minutes with dantrolene sodium 0.5 mg/kg body weight. This pig died due to the irreversible progress of the MH crisis. Both the time elapsed before treatment and the dose of dantrolene sodium administered are important.

As stated before, temperature elevation is a late symptom of MH. Furthermore, dissipation of heat is hampered due to the periferal vasoconstriction[1]. This is caused by the increased output of catecholamines during the MH crisis. This periferal vasoconstiction contributes to the increase of core temperature. Periferal vasoconstriction is evident from the cyanosed-mottled skin.

The cyanosis of the skin points indirectly to a discrepancy of oxygen uptake and -consumption. The amount of oxygen transported depends on the cardiac output and the difference between arterial- and mixed venous oxygen content. Studies on MH induced in pigs indicate two-fold increase of cardiac output[4]. Furthermore, oxygen extraction from blood supplying skeletal muscle masses increases during the MH crisis[5]. Skeletal muscle oxygen consumption increases about 2-3 times as compared to the oxygen

consumption at rest[5]. This means that the oxygen flux during an MH crisis is increased 4-6 times compared to the oxygen consumption at rest.
The respiratory quotient (RQ) during an MH crisis is about one: we may consider the amount of oxygen consumed to be equal to the amount of carbon-dioxyde produced[6].
The observation of a cyanosed mottled skin is the result of both the increased release of catecholamines and the increased oxygen utilisation leading to arterial desaturation.

The signs of hypermetabolism can be explained on the basis of the results of our pig studies. During the MH crisis we observed an accumulation of calcium in the mitochondria[7]. This occurs at the expense of the energy provided by the oxydation of substrates feeding the oxydative phosphorylating process. Furthermore, the uptake of calcium prevents the phosphorylation of ADP to ATP[8]. So, the presence of calcium is detrimental regarding two aspects: energy is used for accumulation of calcium and phosphorylation of ADP to ATP is hampered[9]. The observed accumulation of calcium in the mitochondria, suggests an increased calcium ion concentration in the myoplasma during an MH crisis. This, in turn stimulates glycogenolysis by activating the phosphorylase system. This leads to the accumulation of glycolytic intermediates.

The increased concentration of calcium ions in the myoplasma is associated with activation of the contractile

system: actine and myosine molecules interact with each other and produce skeletal muscle rigidity. In physiological conditions these calcium ions are removed from the cytoplasma back into the sarcoplasmic reticulum (SR). However, apparently the SR can not cope with this increased amount of calcium ions, as the rigidity persists during the MH crisis. Furthermore, the precipitous decrease of energy-rich phosphates, like phosphocreatine (PCr) and ATP, leads to a rigor-mortis like condition.

The site of origin of the calcium ions during an MH crisis is still an enigma. Whether the sarcolemma or the SR are involved in the genesis of the MH crisis is not yet established. Results in favour of a halothane induced effect on a defective sarcolemma in MH susceptible pigs have emerged [10]. In human skeletal muscle obtained from an MH susceptible subject, these findings have not yet been verified. Due to the big gradient of calcium ions across the sarcolemma, the involvement of the sarcolemma in MH is an attractive hypothesis.

Regarding the screening for MH susceptibility in humans, we still have to rely on an in vitro contracture test of a skeletal muscle specimen [1,2]. The evaluation of a proposed less invasive test did not prove to be useful as a screening test [11]. This test, the ATP depletion test in platelets exposed in vitro to halothane [12], provided comparable data in platelets obtained form MH susceptible subjects and controls. Furthermore, halothane inhibited

to the same extent the incorporation of adenine into the platelets[11], this contrast to findings reported by others[13].

The evaluation of skeletal muscle metabolites in humans susceptible to MH and in controls, did not show significant differences in metabolite values measured (ref. chapter 9). This finding is in contrast to the results obtained in our pig studies. As discussed in chapter 9, a possible factor creating the differences in the porcine model might have been the greater stress-susceptibility of the MH-susceptible pigs. Our findings in humans, showing no significant differences in metabolites, contribute to the theory that malignant hyperthermia in humans is a pharmacogenetic disorder, without pre-existent changes or abnormalities. This is in line with the clinical manifestation of MH: the MH susceptible subjects do not show specific abnormalities, neither on physical examination nor on histological examination.

The increased incidence of MH in Anglo-Saxon countries is remarkable. A factor contributing to this phenomenos is the generous use of volatile anesthetics in those countries, in contrast to the Continent. Furthermore, a possible "founder" effect may contribute to the higher incidence of MH in the U.S.A., Canada, Australia and South-Africa. The incidence of an inherited disease in countries predominantly inhabited by immigrants may increase due to the limited number of humans, composing the genetic pool of the next generations.

Regarding the MH crisis, one may question whether an MH crisis is really a specific reaction of MH. As has been stated in chapter 2.5., other syndromes also produce hyperthermic reactions. The pathogenesis however, may be identical: the heat production may be the result of a finally common disturbance at the cellular level. In heat-stroke the increased temperature causes damage to the cellular membranes. Secondary to this damage an influx of extra-cellular ions, especially calcium, will occur into the cytoplasma. As stated before, this increased concentration of calcium increases oxygen consumption of the mitochondria, producing additional heat. These processes contribute to the irreversible state of a heat-stroke and MH crisis. In this respect, study of the MH syndrome has contributed to our understanding of the syndrome of heat-stroke. Furthermore, MH is a clinical expression of the effects of calcium ions on the mitochondrial functioning.

1. Gronert G A. Malignant hyperthermia. Anesthesiology 1980: 53, 395-423.
2. Nelson T E, Flewellen E H. Current concepts, the malignant hyperthermia syndrome. N Engl J Med 1983: 309, 416-418.
3. Verburg M P, Oerlemans F T J J, Van Bennekom C A, Gielen M J M, De Bruyn C H M M, Crul J F. In vivo induced malignant hyperthermia in pigs. I.Physiological and biochemical changes and the influence of dantrolene sodium. Acta Anaesthesiol Scand 1984: 28, 1-8.
4. Lucke J N, Hall G M, Lister D. Porcine malignant hyperthermia. I. Metabolic and physiological changes. Br J Anaesth 1976: 48, 297-304.
5. Gronert G A, Heffron J J A, Midel J H, Theye R A. Porcine malignant hyperthermia: the role of skeletal muscle in increased oxygen consumption. Can Anaesth Soc J 1977: 24, 103-109.
6. Hall G M, Lucke J N, Orchard C, Lovell R, Lister D. Effect of dantronele on leg metabolism in porcine malignant hyperthermia. Anaesthesia 1982: 37, 1167-1170.
7. Stadhouders A M, Viering W A L, Verburg M P, Ruitenbeek W, Sengers R C A. In vivo induced malignant hyperthermia in pigs. III. Localization of calcium in skeletal muscle by means of electronmicroscopy and microprobe analysis. Acta Anaesthesiol Scand 1984: 28, 14-26.
8. Lehninger A L. Biochemistry. 2nd ed New York: Worth,

1977: 531-533.

9. Ruitenbeek W, Verburg M P, Janssen A J M, Stadhouders A M, Sengers R C A. In vivo induced malignant hyperthermia in pigs. III. Metabolism of skeletal muscle mitochondria. Acta Anaesthesial Scand 1984: 28, 9-13.
10. Gallant F M, Gronert G A, Taylor S R. Cellular membrane potentials and contractile threshold in mammalian skeletal muscle susceptible to malignant hyperthermia. Neurosci Lett 1982: 28, 181-186.
11. Verburg M P, Van Bennekom C A, Oerlemans F T, De Bruyn C H M M. Malignant hyperthermia: adenine incorporation and adenine metabolism in human platelets, influenced by halothane. In: De Bruyn C H M M, eds. Purine metabolism in man--IV, part B: Biochemical, Immunological and Cancer research. New York, Plenum, 1984: 443-446.
12. Solomons C C, McDermott N, Mahowald M. Screening for malignant hyperthermia with a platelet bioassay (letter) N Engl J Med 1980: 303-642.
13. Solomons C C, Tan S, Aldrete J A. Platelet metabolism and malignant hyperthermia. In: Aldrete J A, Britt B A eds. Malignant hyperthermia, second international symposium. New York: Grune and Stratton, 1978: 221-225.

Malignant hyperthermia (MH) is due to its rarety seldom encountered by physicians. Most described case reports on MH are based on observed MH reactions related to general anaesthesia. Still, an anaesthetist may meet only 1 to 5 cases of MH in his or her professional life. In this respect we thought it appropriate to present an extensive review of the clinical manifestations of MH as well as the pathophysiological and aetiology of MH (chapter 2 and 3 respectively).

Screening for MH is a complex matter. Various methods have emerged and vanished. A review of the existing screening methods is given in chapter 4, and a conclusion is given about the best methods to be employed.

These chapters (2, 3 and 4) were also felt necessary in relation to our experimental work, described in chapters 5-10. The experimental work can be divided in an animal study on pigs and human studies. The animal study on pigs was performed in order to gain a better insight into the pathophysiological and biochemical changes during an induced MH crisis and reversal with dantrolene sodium. Furthermore, an attempt was made to provide evidence for the involvement of calcium in the aetiology of MH.

The study on humans was performed in order to evaluate a less invasive screening method. Studies have been

performed on platelet ATP depletion and adenine incorporation, chapter 8. Based on the results from our pig studies we have evaluated skeletal muscle metabolites from MH susceptible humans, in order to find possibly a less invasive test, chapter 9. In chapter 10, the results of the in-vitro contracture test in our hands is discussed, as well as the results of the use of an amide type local anaesthetic in a regional anaesthetic block.

Maligne hyperthermie (MH) is een zeldzaam voorkomend erfelijk ziektebeeld. De meeste voorvallen zijn beschreven in relatie tot het toedienen van een algemene anaesthesie. Tijdens haar/zijn praktische werkzaamheden als anaesthesist(e) zal elke anaesthesist(e) slechts 1 tot 5 gevallen van MH kunnen meemaken. Derhalve hebben wij het nodig geacht, wegens de onbekendheid met dit ziektebeeld, om een uitgebreid literatuuroverzicht te geven van de klinische verschijnselen en de pathophysiologie en de aetiologie van MH (hoofdstuk 2 en 3).

Aangezien het onderzoek naar de erfelijke aanleg voor MH een complexe zaak is, hebben wij een overzicht gegeven van de huidige methodes (hoofdstuk 4). Bovendien is een aanbeveling gegeven over de beste onderzoeksmethodes.

De voorgaande hoofdstukken werden nodig geacht in verband met ons experimenteel werk, zoals beschreven in de hoofdstukken 5 tot en met 10. Het experimentele werk kan verdeeld worden in een dierexperimenteel gedeelte en een gedeelte gewijd aan onderzoek bij mensen. Het dierexperimenteel onderzoek is verricht om het inzicht te verdiepen in de pathophysiologische en biochemische veranderingen tijdens een MH aanval. Bovendien is het effect van dantroleen op de MH aanval bestudeerd, alsmede de rol van calcium in het ontstaan van een MH aanval.

Het onderzoek bij mensen is gedaan ter evaluatie van een minder belastende onderzoeksmethode. Dit onderzoek had betrekking op het effect van halothane op de afname van adenosine triphosphaat (ATP) en op de inbouw van adenine in de bloedplaatjes van MH gevoelige personen en niet-MH gevoelige personen (hoofdstuk 8).
Gezien de resultaten van het onderzoek aan varkens met betrekking tot enkele metabolieten in de skeletspier, is dit onderzoek ook verricht bij MH gevoelige personen. Dit ter evaluatie van een mogelijke, minder belastende, wijze van onderzoek naar de aanleg van MH (hoofdstuk 9).
In hoofdstuk 10 is een overzicht van onze resultaten gegeven van de huidige, als meest betrouwbaar geachte, methode om MH gevoeligheid vast te stellen. Bovendien zijn de resultaten beschreven van een regionale anaesthesie techniek, met een amide type locaal anaestheticum, ten behoeve van het nemen van een spierbiopsie.

APPENDIX

A PROTOCOL FOR THE INVESTIGATION OF MALIGNANT HYPERPYREXIA (MH) SUSCEPTIBILITY BY THE EUROPEAN MALIGNANT HYPERPYREXIA GROUP.

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An European Malignant Hyperpyrexia Group has been formed to facilitate exchange of information between centres performing *in vitro* muscle testing for malignant hyperpyrexia susceptibility. Data has been collected according to a protocol agreed by the Group. Based on these results, test criteria have been established to allow the following diagnoses to be made: MH susceptible (MHS); MH normal (MHN) or equivocol (MHE). It is accepted that MHE classified patients will be under permanent review pending the collection of further data.

In April, 1983, doctors from 8 European Countries met in Lund, Sweden. They were all actively involved in screening patients for malignant hyperpyrexia susceptibility (MHS). An European Malignant Hyperpyrexia Group with the following aims was established:

- to provide a forum for discussion between the various European centres.
- to standardize the investigation of MH subjects to allow comparisons between centres.
- to establish a common data bank.
- to allow for combined research facilities.

An essential first stage was to establish a specific protocol for *in vitro* screening tests similar in principle to that suggested by Rosenberg & Reed (1983). However, as members of the European MH Group invariably use a halothane contracture test, this test was included.

The full protocol is as follows :

1. The biopsy should be performed on the quadriceps muscle and the samples taken from the region of the muscle which includes the motor point.
2. Biopsy specimens suitable for <u>in vitro</u> investigations should measure 15-25 mm in length with a thickness of 2-3 mm.
3. The muscle should be placed immediately in carboxygenated Krebs-Ringer solution with a composition of :

NaCL	118.1 mM
KCL	3.4 mM
$MgSO_4$	0.8 mM
$KH_2P\ O_4$	1.2 mM
Glucose	11.1 mM
$NaHCO_3$	25.0 mM
$CaCl_2\ 6H_2O$	2.5 mM
pH	7.4 mM

4. The muscle should be transported to the laboratory in Krebs-Ringer solution at ambient temperature. In the laboratory is should be kept at roomtemperature and carboyxgenated.
5. The time from biopsy to the completion of the tests should not exceed 5 hours.
6. The tests should be performed at 37 ^{o}C in a tissue bath perfused either intermittently or continuously with Krebs-Ringer solution and continuously oxygenated with carbogen.
7. The muscle specimen should be electrically stimulated

with a 1 ms supra-maximal stimulus at a frequency of 0.2 Hz.

8. Three tests should be performed each on a fresh specimen. The tests include: a static caffeine test, a static halothane test and a dynamic halothane test (see figure 1)

9. For the static tests the muscle tension should be gradually increased to produce a reasonable twitch height; this will usually occur with 2 g preload.

10. The static cummulative caffeine test and measurement of the caffeine threshold

    The concentration of caffeine in the tissue bath should be increased step-wise as follows: 0.25; 0.5; 1.0; 1.5; 2.0; 3.0; and 4.0 mM.

    Each successive concentration of caffeine should be administered as soon as the maximum contracture plateau, induced by the previous concentration of caffeine, has been reached, or after exposure to the caffeine concentration for 3 minutes if no contracture occurs.

    The result of this test will be reported as a caffeine threshold which is defined as the lowest concentration of caffeine which produces a sustained rise of at least 0.2 g in baseline tension.

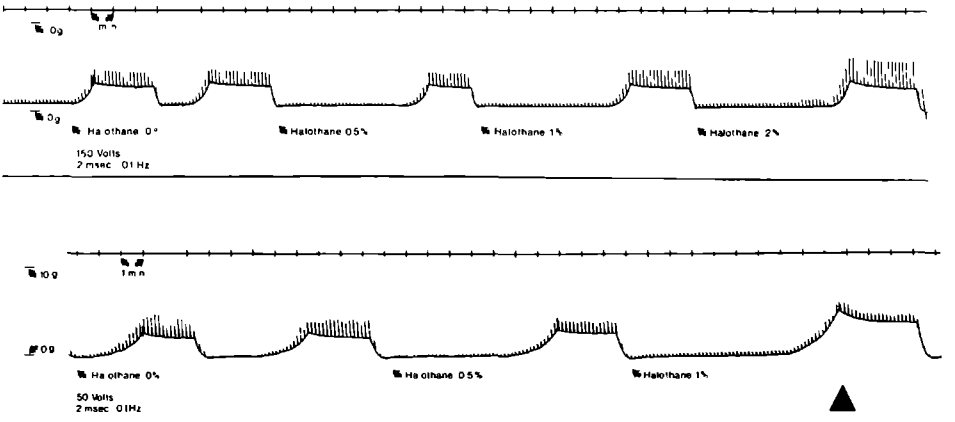
11. The static halothane test and measurement of static halothane threshold

    The halothane threshold is obtained using the nominal

halothane concentrations 0.5; 1.0; 2.0 and 3.0% v/v. The measurement of the threshold is similar to (10) above.

12. <u>The dynamic halothane test and measurement of dynamic halothane threshold</u>

After three minutes exposure to halothane, the muscle is stretched at a constant rate of 4 mm min for 1.5 min and held at this new length for 1 min. The stretching process is then reversed. At each cycle the halothane concentration is increased from 0.5; 1.0; 2.0 to 3.0% v/v. The dynamic halothane threshold is defined as the concentration of halothane which produces a sustained rise of at least 0.2 g in the muscle tension compared with a prehalothane control measured at the point shown in figure 1. (▲)



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Het laboratorium voor Anthropogenetica is met zijn staf zeer waardevol geweest in het exploreren en exploiteren van overzeese bronnen van maligne hyperthermie. De heer F.T.J.J.Oerlemans en de heer C.A. van Bennekom zijn voortdurend belaagd geworden met verzoeken tot analyse van spierextracten en bloedplaatjes.

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In de beginfase van het onderzoek is veel steun verkregen van het laboratorium Haematologie St.Radboud Ziekenhuis, hoofd Dr. H.Wessels; met name van de heer P.Linsen. Het dierenlaboratorium te Nijmegen, hoofd Dr. Van der Gulden, is door de deskundige medewerking van de heer T.Arts van wezenlijk belang geweest voor het slagen van het onderzoek. Het laboratorium voor kindergeneeskunde, St.Radboud Ziekenhuis, is dankzij de medewerking van Dr. J.L.Willems behulpzaam geweest met de serologische bepalingen.

De collegae anaesthesisten van het De Wever Ziekenhuis hebben mij de physieke mogelijkheid gegeven om dit onderzoek te verrichten. Mevrouw R.Clignet, bibliothecaresse De Wever Ziekenhuis, heeft zeer nauwgezet de literatuuraanvragen verzorgd. Mejuffrouw J.Dons heeft schier onvermoeibaar de steeds zich wijzigende manuscripten vervaardigd.

CURRICULUM VITAE

De schrijver van dit proefschrift is geboren op 6 april 1948 te Geersdijk (gemeente Wissenkerke) als zoon van Adriaan Pieter Verburg en Christina Hendrika Priester. Lager onderwijs werd genoten aan de Hervormde School voor lager onderwijs te Terneuzen en het MULO-B-diploma werd behaald in 1964 aan de Chr. MULO te Terneuzen. Het diploma H.B.S.-B werd behaald aan het Petrus Hondius Lyceum te Terneuzen in 1967.
Het artsdiploma werd behaald te Utrecht in januari 1974.
Tijdens de studentenperiode actief wedstrijdroeier geweest, Senior-A en bestuurslid van de A.U.S.R. Orca.
Na het volgen van diverse klinische stages, de tropencursus te Amsterdam en waarnemingen bij huisartsen, volgde de uitzending naar Ethiopie 1976-1977. Het E.C.F.M.G.-examen werd behaald in september 1975. De opleiding tot anaesthesioloog werd gevolgd te Nijmegen (Prof.Dr. J.F.Crul). Sedert 1981 werkzaam als anaesthesioloog aan het De Weverziekenhuis te Heerlen.
In augustus 1974 is hij gehuwd met Theresia George Maria König, hetgeen heeft mogen leiden tot de geboorte van Marijn en Suzanne.

I.

Verder bewijs is nodig om een membraandefect te veronderstellen als grondslag voor maligne hyperthermie.

(dit proefschrift)

II.

Gezien de mogelijkheid tot snelle diagnostiek van een maligne hyperthermie aanval met een capnometer, dient dit apparaat tot de standaarduitrusting van elk anaesthesie toestel te behoren.

(dit proefschrift)

III.

Er bestaat een toenemende behoefte aan een niet-invasieve diagnostische methode ter vaststelling van gevoelig zijn voor maligne hyperthermie.

(dit proefschrift)

IV.

Bij het aanwezig zijn van adequaat opgeleide anaesthesie-verpleegkundigen zal het invoeren van het systeem van "een anaesthesioloog per tafel" nauwelijks leiden tot een verhoging van de veiligheid van de patient en daardoor de gezondheidszorg onnodig duur maken.

V.

De hotelfunctie van ziekenhuizen vertoont een discrepantie tot de verpleegprijs.

VI.

Het gebruik van dezelfde kleur blauwe verf (Pantone^{R} 292) ter identificatie van cylinders met lachgas in de U.S.A. en zuurstof cylinders in Europa, zal leiden tot een toename van onnodige calamiteiten bij internationale hulpverlening.

(Int Anesthes Clin 1982, Vol 20, no 3, 206)

VII.

Het heffen van invoerrechten op medicijnen en het hanteren van het hoge B.T.W.-tarief op medische apparatuur, plaatst

de overheid in een dubieus licht in haar streven tot een beheersing van de kosten in de gezondheidszorg.

VIII.

De kwaliteit van de geneeskunde is niet alleen afhankelijk van de verrichtingen die men doet, doch ook van de verrichtingen die men bewust niet doet.

IX.

Blijkbaar bestaat er een overeenkomst tussen de Berlijnse Muur en de Heyendaalse weg te Nijmegen met betrekking tot de scheiding tussen de Faculteit der Geneeskunde en die der Wiskunde en Natuurwetenschappen.

X.

Gezien de ernstige hypokalemieën, veroorzaakt door Hygroton^{R}, dient dit medicament verboden te worden.

XI.

Het tweeslachtig beleid van de Medische Faculteit der Rijks Universiteit Limburg ten aanzien van de eerste- en tweede lijns gezondheidszorg, frustreert de gewenste samenwerking tussen de tweede lijns gezondheidszorg en de voornoemde Medische Faculteit.

XII.

Een optimale opvang van de poly-traumatisé dient te geschieden door een team bestaande uit: anaesthesiologen, chirurgen, neurologen, neurochirurgen èn orthopaeden.

XIII.

De schooltijden van het basisonderwijs vertonen ongewild een grote mate van overeenkomst met een werkverschaffingsproject voor de ouders of verzorgers met betrekking tot het brengen en halen van hun kinderen.